Signaling mechanisms of Down syndrome cell adhesion molecule in presynaptic arbor size control

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Neuroscience)
in the University of Michigan
2016

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DEDICATION

To my parents, who taught me the value of education and instilled in me a love of nature.

ACKNOWLEDGEMENTS

I would like to thank my research mentor, Dr. Bing Ye, for the opportunities, guidance, and patience he has provided me throughout my time at the University of Michigan. From the time I arrived in Dr. Ye's lab, I was given thorough hands-on training and was taught to pursue the highest standards of research. Under Dr. Ye's instruction, my critical thinking, scientific writing, and research skills have grown exponentially, and I have benefitted tremendously from his incredible breadth of knowledge as well as many in-depth conversations on the topic of neuroscience. I hope that I will always approach neuroscience with the same excitement and curiosity as Dr. Ye.

I would also like to thank my thesis committee members, Drs. Peter F. Hitchcock, Edward L. Stuenkel and Michael A. Sutton. Their support, advice, and scientific guidance has proven invaluable to the creation of this dissertation. I am grateful for their generosity in providing letters of reference, feedback on scientific writing, and overall encouragement. In particular, I would like to thank Dr. Hitchcock for writing an excellent question for my preliminary exam and for facilitating an overall excellent prelim experience. I would also like to thank Dr. Stuenkel for his friendship, sense of humor, and perspective throughout my time at the University of Michigan as well as for the opportunity to rotate and do continuing experiments in his laboratory.

For their generous gifts of reagents, I would like to thank Drs. Tzumin Lee, S. Lawrence Zipursky, Peter Todd, Kenneth Kwan, and Yusuke Ohba.

I would like to thank the many members of the Ye Lab and the University of Michigan community whom I have worked with throughout my Ph.D. work. First and foremost, I would like to thank Dr. Jung Hwan Kim, with whom I collaborated to produce the Dscam-Abl story. He performed the in vivo drug treatment assays and experiments presented in Figures 2.9-2.12 in this dissertation. I would also like to thank Dr. Kim for his friendship and generosity with his time and advice. Under his careful tutelage I have learned many valuable lessons and techniques, including Western blotting, co-immunoprecipitation, and cell culture techniques. Additionally, I would like to thank the many past and present members of the Ye lab: Wei Zhou, Limin Yang, Megan Stewart, Mouna Bazzi, Xin Wang, Laura Essex, Lindsey Cregan, Rosemary Coolon, Takuya Kaneko, Ann Marie Macara, Macy Zhang, Hao Lui, Yujia Hu, and Tao Yang.

Throughout my PhD I have benefitted from the support and resources provided by the Neuroscience Program. In particular I would like to thank Valerie Smith, Rachel Harbach, and Dr. Audrey Seasholtz for their advice and assistance.

Finally, I would like to thank my family, friends, and my fiancée, Dr. Brian Kearns, for their unwavering support during my PhD. Without their companionship, pep-talks, advice, and occasional gifts of sustenance, this dissertation would not have been possible.

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LIST OF ABBREVIATIONS

Abbreviaton	**Stands for**
Dscam	Down syndrome cell adhesion molecule
Abl	Abelson tyrosine kinase
APP	Amyloid Precursor Protein
Appl	Amyloid precursor protein-like
FMR1	Fragile X Mental Retardation 1
FMRP	Fragile X Mental Retardation Protein
NMJ	Neuromuscular Junction
VNC	Ventral Nerve Cord
Wg	Wingless
Gbb	Glass bottom boat
BMP	Bone Morphogenetic Protein
TGFβ	Transforming Growth Factor-β
DFz2	DFrizzled2
Hiw	Highwire
C4da	Class 4 dendritic arborization
CNS	Central Nervous System
Ig	Immunoglobulin
ORNs	Olfactory Receptor Neurons
da	dendritic arborization
DCC	Deleted in Colorectal Cancer
BCR	Breakpoint Cluster Region
S2	*Drosophila* Schneider 2
MARCM	Mosaic Analysis with a Repressible Cell Marker
Pickles2.31	Phosphorylation indicator of CrkL en substrate number 2.31
FRET	Förster resonance energy transfer
UAS	Upstream activating sequence

Chapter 1 Introduction

1.1 The importance of the development of proper dendritic and axonal morphology

The development of proper neuronal morphology is critical for nervous system function. The brain is composed electrically excitable cells, neurons, which transmit signals through both electrical and chemical means. Neurons are typically composed of two distinct polarized compartments, the dendrites and the axon. Dendrites receive and process signals, while axons take that information and pass it on to other cells. To perform these two distinctive functions, axons and dendrites each have characteristic machinery and morphology. Diverse neuronal morphologies reflect the diverse functions that specific neuronal types must carry out, and defects in the development of neuronal morphology can lead to defective nervous system function. On the dendritic side of the neuron, defects in dendrite and dendritic spine morphology have been linked to many developmental disorders, where characteristic spine defects are hypothesized to impact information processing and synaptic transmission (Kaufmann and Moser, 2000). On the axonal side of the neuron, defects in axon growth and pathfinding may lead to improper synaptic connections that interfere with circuit development and function.

1.1.1 Mechanisms underlying the development of the presynaptic arbors

Several steps during development determine final axonal morphology. First, the axon must be properly specified and differentiated from the dendrites. Second, the axon enters a phase of rapid growth, during which it elongates toward its target. Third, upon reaching its target the axon forms local elongations, or presynaptic arbors, which interface with the target cell or cells.

Fourth, synapses form at sites throughout the presynaptic arbor, allowing for neural transmission. This dissertation focuses on the third step of axon development, the development of the presynaptic arbor. These processes are best studied in the neuromuscular junction (NMJ), where peripherally located motor neuron presynaptic arbors impinge on postsynaptic muscle fibers.

In mammals, NMJ development begins with the prepatterning of acetylcholine receptors (AChRs) to the central band of the developing muscle. Following interaction of the motor neuron axon with the muscle, postsynaptic AChR clusters form to perfectly appose the sites of motor axon impingement. These clusters form both by recruiting existing AChRs from the prepatterned band and by targeted accumulation of newly-generated AChRs. Trans-synaptic signals between the pre-synaptic motor axon and the post-synaptic myotubule coordinate these steps. A number of key molecular players in these events have been uncovered, including agrin, MuSK, Lrp4, and Dok-7.

Agrin is a heparin sulfate proteoglycan that is released from the motor neuron onto the muscle membrane. While not required for AChR prepatterning (Lin et al., 2001), agrin acts as a motor neuron-derived signal that organizes many aspects of NMJ development (Rupp et al., 1991; Smith et al., 1987). Exposure of the postsynaptic muscle to agrin leads to phosphorylation of the AChR-β subunit, which triggers the redistribution of AChRs into clusters at the sites of motor neuron impingement (Wallace, 1986). Loss of agrin also leads to presynaptic defects in the NMJ. Most notably, motor axons in agrin-deficient mice leave the nerve tract, as in wildtype mice, but then fail to form presynaptic arbors terminating on myotubes, instead running parallel to the myotubules without branching (Gautam et al., 1996). Thus, agrin is important for the proper growth and patterning of both the pre- and post-synaptic sides of the NMJ.

On the muscle side of the synapse, agrin's receptor, muscle-specific kinase (MuSK) accumulates along with AChRs to the central band of the developing muscle. MuSK is a transmembrane receptor tyrosine kinase that is both necessary and sufficient to induce the entire post-synaptic apparatus (Kim et al., 2008; Sander et al., 2001), and MuSK mutants lack AChR clusters. Like agrin mutants, motor axons in mice lacking MuSK grow beyond the central region of the muscle and extend along the entire myotubule, but no pre-synaptic structures can be appreciated (DeChiara et al., 1996). Interestingly, however, MuSK does not bind directly to agrin. The interaction between agrin and MuSK is mediated by low-density lipoprotein receptor-related protein 4 (Lrp4), an agrin-binding protein that forms a receptor complex with MuSK (Kim et al., 2008; Zhang et al., 2008). Loss of Lrp4 causes complete abrogation of NMJ development if deleted in both the motor neuron and the muscle (Weatherbee et al., 2006).

Activation of the MuSK-Lrp4 complex by agrin leads to the phosphorylation of a non-catalytic cytoplasmic adaptor protein, Downstream of tyrosine kinases-7 (Dok-7), which serves as a promiscuous adaptor protein for tyrosine kinases. Dok-7 mutants lack NMJs, and have a similar phenotype to Argin-, MuSK- and Lrp4-deficient mice in that the motor axons fail to form presynaptic specializations and instead grow along the entire length of the myotubule (Okada et al., 2006). Phosphorylation of Dok-7 creates binding sites for v-crk sarcoma virus CT10 oncogene homolog (Crk) and Crk-like (Crk-L). Newborn mice that lack Crk and Crk-L specifically in muscle die at birth due to respiratory failure, and closer inspection of their NMJs reveals a variety of defects. Although NMJs form in these mutants, motor axons are not confined within a narrow band on the myotubule and instead spread out over a wider region. In addition, synaptic size is reduced in mice that lacked Crk and Crk-L in muscle and motor axons frequently contacted AChR clusters but then continued to grow. This was in contrast to wild-type mice,

where motor axons terminated on AChR clusters (Hallock et al., 2010). Thus, Crk and Crk-L may play a role in regulating the size and patterning of NMJs in mammals.

Patterning and growth of NMJs vertebrates may also be regulated by Wnt signaling in vertebrates. Wnts are known to be involved in axon pathfinding and synaptogenesis, and the roles of Wnts in vertebrate NMJ development have been best studied in zebrafish. As in mice, deletion of the zebrafish ortholog of mammalian MuSK, unplugged, leads to a loss of AChR prepatterning and impairs guidance of motor axons to the central band of the myotubule. In addition to MuSK, Wnt11r is required to confine developing motor axons to the central region of the myotubule and for prepatterning of AChRs. Wnt11r binds to MuSK and is thought to signal through downstream dishevelled (Jing et al., 2009). Thus, in vertebrates, it seems that Wnts are important for controlling NMJ size and patterning.

In addition to mammalian studies, analysis of NMJ development in *Drosophila* has contributed much to our understanding of presynaptic arbor development. The advantages of using *Drosophila* include fast generation times, stereotyped morphology, and an extensive genetic toolkit. In *Drosophila*, NMJ presynaptic arbor development is regulated by trans-synaptic signals, including Wingless (Wg), which belongs to the Wnt family, and Glass bottom boat (Gbb), part of the bone morphogenetic protein (BMP) and transforming growth factor-β (TGFβ) family. Both Wg and Gbb signal to the nucleus, presumably leading to changes in gene expression. Wg is secreted by motor neuron axon terminals, where is subsequently binds to the Wg receptor, DFrizzled2 (DFz2) on the post-synaptic membrane. Loss of both Wg and DFz2 lead to defects in presynaptic arbor growth, and flies mutant for either protein exhibit smaller NMJ presynaptic arbors with fewer boutons (Mathew et al., 2005; Packard et al., 2002). These defects can be rescued by postsynaptic expression of DFz2, suggesting that a yet unidentified

retrograde signaling mechanism allows the muscle to stimulate presynaptic arbor growth in response to Wg secretion (Ataman et al., 2006). In contrast, release of Gbb from the postsynaptic muscle stimulates the BMP/TGFβ receptors Wit, Tkv and Sax on the presynaptic motor neuron which leads to phosphorylation of MAD. Phospho-MAD then translocates to the motor neuron nucleus, presumably leading to changes in gene expression that affect presynaptic arbor growth (Marques, 2005).

Although both Wg and Gbb signal to the nucleus, changes in presynaptic arbor growth are ultimately transduced by proteins that lead to local changes in the cytoskeleton and membrane. Previous studies have demonstrated roles for actin regulators like Nervous Wreck (Coyle et al., 2004) and WAVE/SCAR complex components (Schenck et al., 2004), microtubule regulators like Shaggy (Franco et al., 2004) and spastin (Trotta et al., 2004), cell adhesion molecules including Fasciclin II and Amyloid Precursor Protein(Ashley et al., 2005), and finally regulators of membrane trafficking (Dickman et al., 2006; Koh et al., 2004). Furthermore, although factors that promote growth are essential to presynaptic arbor development, it is also important to prevent excessive growth. Highwire (Hiw), an E3 ubiquitin ligase, is an evolutionarily conserved negative regulator of presynaptic arbor growth (Wu et al., 2005). Loss of Hiw function leads to huge increases in presynaptic arbor growth, resulting in excesses of both presynaptic arbor branches and boutons. Furthermore, Hiw requires Wallenda (Wnd), a mitogen-activated protein kinase (MAPK) kinase kinase (MAPKKK) (Collins et al., 2006). The role of Hiw in regulating presynaptic arbor growth suggests that local control over protein degradation and synthesis may be essential to regulating presynaptic arbor growth, an idea that is supported by a number of previous studies (Aravamudan and Broadie, 2003; Sigrist et al., 2000; Speese et al., 2003).

Although less studied, research on centrally located *Drosophila* presynaptic arbors suggest that some mechanisms that control NMJ presynaptic arbor development may be shared. In particular, these studies have been performed using the Class IV dendritic arborization (C4da) neurons. These multidendritic neurons are nociceptors that mediate larval avoidance of potentially harmful stimuli (Hwang et al., 2007). The cell body and dendrites of each C4da neuron are located in the larval body wall, with the dendrites fanning out to cover the dorsal region of the body wall. These neurons are segmentally repeated, and their axons project to the ventral nerve cord (VNC) at regular intervals. Upon reaching the VNC, C4da axon terminals branch into anterior, posterior, and contralateral projections that together form a ladder-like structure. These axon terminals have previously been shown to be presynaptic terminal arbors based on localization of the presynaptic marker synaptotagmin (Kim et al., 2013). This system can be manipulated with numerous genetic tools, including mosaic analysis with a repressible cell marker (MARCM). Using recombination mediated by a heat-activated flippase at specified sites, this technique allows single, homozygous mutant neurons to be labeled in an otherwise heterozygous background. This technique is important because it allows single C4da presynaptic arbors to be labeled and measured, in addition to allowing investigation of the cell-autonomous consequences of loss-of-function of mutations that are lethal in the whole fly.

Previous studies that employed the C4da neuron system have shown that Hiw is also an important negative regulator of C4da presynaptic arbor growth and that, furthermore, Hiw similarly requires Wnd to regulate presynaptic arbor growth (Wang et al., 2013). These findings suggest that similar mechanisms may direct presynaptic arbor growth in NMJ axon terminals and C4da presynaptic arbors. In this dissertation, I employ the C4da neuron system to study

presynaptic arbor growth as it relates to common causes of intellectual disability, Down syndrome and Fragile X syndrome.

1.1.2 The biology of Down syndrome and Fragile X syndrome

The most common genetic cause of intellectual disability is Down syndrome, while the most common inherited cause of intellectual disability is Fragile X syndrome. Down syndrome occurs in 1 in 850 to 1110 live births (Besser et al., 2007). Fragile X syndrome occurs in approximately 1 in 3,600 to 4,000 males and 1 in 4,000 to 6,000 females (Turner et al., 1996). Down syndrome is caused by the duplication of human chromosome 21 (Jacobs et al., 1959; Lejeune et al., 1959) and is typified by delays in growth, characteristic facial features, and intellectual disability. In addition, individuals with Down syndrome are at a higher risk of developing Alzheimer's disease, leukemia, congenital heart defects, and thyroid problems. The increase in copy-number of chromosome 21 results in the increased expression of a number of genes, but which of these are responsible for the diverse traits seen in Down syndrome is still a subject of much debate.

The early assertion that chromosome band 21q22 is "pathogenic" for Down syndrome (Niebuhr, 1974) laid the foundation for the investigation of the biochemical and developmental processes by which the characteristics of Down Syndrome arise. Subsequent studies of individuals with partial trisomies of chromosome 21 sought to determine which specific regions were responsible for particular Down syndrome traits (Epstein et al., 1991; Korenberg et al., 1994). Early analysis suggested that dosage alteration of an extended region of chromosome 21, termed the "Down syndrome critical region" was responsible for the most salient characteristics of Down syndrome (Korenberg et al., 1994; Rahmani et al., 1989). However, since human trisomies are rare, these phenotypic maps were of low resolution, preventing effective analysis at

the required level of single genes (Korbel et al., 2009). More recent analysis using state-of-the-art genomics and large panels of partial trisomy patients argues against the existence of a single critical region in Down syndrome, but suggests the importance of specific genes to particular Down syndrome phenotypes (Korbel et al., 2009).

Of particular interest to the field of neuroscience is analysis of genes responsible for one of the most disabling aspects of Down syndrome, intellectual disability. Intellectual disability and atypical central nervous system (CNS) development are characteristic of Down syndrome, with patient IQ declining in the first year of life and reaching 25-55 in adulthood (Pennington et al., 2003). Down syndrome patients have specific cognitive profiles which include "uneven" working memory deficits, wherein verbal short-term memory is more impaired than visuospatial short-term memory, impaired long-term explicit verbal and visual object association memory, poor expressive and receptive language, and impaired reading ability (Dierssen, 2012). Korbel et al. (2009) suggest that there may be a "critical region" for Down syndrome intellectual disability phenotypes, which contains several clustered genes including RCAN1, DYRK1A, Down syndrome cell adhesion molecule (Dscam), SNF1LK, TMEM1, PFKL, S100β, and possibly Amyloid Precursor Protein (APP). Of these, Dscam and APP are of particular interest because of their known roles in nervous system development and disease.

As predicted based on the increased copy number of the Dscam gene in Down syndrome, Dscam is overexpressed in the brains of Down syndrome patients (Saito et al., 2000). Dscam levels have also been shown to be elevated in brain tissue of patients with intractable epilepsy, where it may contribute to mossy fiber sprouting (Shen et al., 2011). In addition, increased levels of Dscam have been seen in postmortem brain tissue of patients with bipolar disorder (Amano et al., 2008). Furthermore, Dscam has been shown to bind to Fragile X Mental Retardation Protein

(FMRP), and work in *Drosophila* models of Fragile X syndrome suggests that FMRP controls Dscam expression such that Dscam expression level may be increased in Fragile X syndrome.

Fragile X syndrome is the most common inherited cause of intellectual disability and is considered to be the most common single-gene cause of autism spectrum disorder. Fragile X syndrome is caused by the expansion of CGG triplet repeats in the 5' untranslated region of the *Fragile X Mental Retardation 1* (*FMR1*) gene on the X chromosome, which leads to silencing of FMRP expression. A CGG copy number of between 6 and 54 repeats is considered normal, while Fragile X syndrome patients typically have over 200 repeats (Fu et al., 1991). CGG repeat expansion leads the appearance of a chromosomal gap or constriction in metaphase spreads which is prone to break under certain conditions, hence the term "Fragile X" (Hecht and Sutherland, 1985). Individuals affected with Fragile X syndrome have intellectual disability, characteristic facial features, and a predisposition to attention deficit disorder and seizures. Furthermore, one-third of individuals with Fragile X syndrome have features of autism spectrum disorder, which impairs social interactions and leads to repetitive behaviors and avoidance of eye contact (Garber et al., 2008). Postmortem analysis of Fragile X syndrome patient brains reveals dense, immature, tortuous dendritic spines (Kaufmann and Moser, 2000). FMRP is a selective mRNA binding protein that associates with up to 4% of mammalian mRNAs (Ashley et al., 1993). FMRP reversibly stalls ribosomes, and hence translation, of its target mRNAs so that without FMRP, the levels of many of these proteins are increased (Darnell et al., 2011). However, it is not clear which of these proteins are relevant to the development of intellectual disability in Fragile X syndrome.

1.1.3 Similarities between Down syndrome and Fragile X syndrome

Interestingly, several lines of evidence point to shared pathogenic mechanisms between Down syndrome and Fragile X syndrome. Importantly, FMRP has been shown to bind to both Dscam and APP mRNAs in the mouse brain, which are both increased and implicated in the intellectual disability phenotype in Down syndrome (Brown et al., 2001; Darnell et al., 2011). In addition, work in our lab and others found that loss of FRMP led to increased Dscam expression in *Drosophila* in vivo (Cvetkovska et al., 2013; Kim et al., 2013). Furthermore, hyperconnectivity between neurons has been reported in mouse models of both Down syndrome and Fragile X syndrome and in human patients with each of these disorders. In Ts65Dn mice, a well-characterized model of Down syndrome, electrophysiological characterization found an increase in associational synaptic connections between pyramidal neurons in the CA3 area of the hippocampus (Hanson et al., 2007).

Similarly, analysis of synaptic connections between layer 5 pyramidal cells in the medial prefrontal cortex of *FMR1* knockout mice, a mouse model for Fragile X syndrome, revealed an increase in connectivity between neighboring pyramidal neurons during development (Testa-Silva et al., 2012). Similar hyperconnectivity has been observed in human patients with Down syndrome and Fragile X syndrome using functional magnetic resonance imaging (fMRI). Anderson et al. observed that although Down syndrome patients show simplified brain network architecture, neighboring brain areas show increased synchronization as compared to healthy controls (Anderson et al., 2013). In autism spectrum disorder patients, several studies have reported hyperconnectivity at the whole-brain and subsystems levels, and found that the level of hyperconnectivity can predict symptom severity (Supekar et al., 2013; Uddin et al., 2013). Although these studies were performed on mixed cohorts of autism spectrum disorder patients,

including patients with and without Fragile X syndrome, the fact that hyperconnectivity scores are able to predict autism spectrum disorder symptoms in isolated brain scans suggests that Fragile X syndrome patients also exhibit hyperconnectivity.

1.1.4 Scope of this dissertation

My dissertation seeks to understand the mechanisms that translate Dscam expression level into changes in presynaptic arbor size. Furthermore, it asks whether these mechanisms can inform the development of potential therapies for both Down syndrome and Fragile X syndrome. It is divided into two parts. The first part reports that Dscam requires a cytoplasmic tyrosine kinase, Abelson tyrosine kinase (Abl), to promote presynaptic arbor growth and that pharmacologically inhibiting this pathway can rescue the morphological consequences of increased Dscam. This work provides proof of principle that knowledge of Dscam signaling mechanisms may be able to inform the development of therapies for Down syndrome and Fragile X syndrome. The second part of this dissertation reports Dscam and Appl, the *Drosophila* homolog of APP, are mutually required and that Appl furthermore requires Abl to promote presynaptic arbor growth. Finally, I show that simultaneous increases in Dscam and Appl act synergistically to promote presynaptic arbor size, suggesting that simultaneous increases in Dscam and APP in Down syndrome or Fragile X syndrome may lead to more severe developmental alterations than an increased levels of either protein alone.

Chapter 2 Dysregulated Dscam levels act through Abelson tyrosine kinase to enlarge presynaptic arbors

2.1 Abstract

Increased expression of Down Syndrome Cell Adhesion Molecule (Dscam) is implicated in the pathogenesis of brain disorders such as Down syndrome (DS) and fragile X syndrome (FXS). Here, we show that the cellular defects caused by dysregulated Dscam levels can be ameliorated by genetic and pharmacological inhibition of Abelson kinase (Abl) both in Dscam-overexpressing neurons and in a *Drosophila* model of Fragile X syndrome. This study offers Abl as a potential therapeutic target for treating brain disorders associated with dysregulated Dscam expression.

2.2 Introduction

Dscam was first identified because it resides in the so-called Down syndrome "critical region", located on chromosome band 21q22 distal to D21S267 (Yamakawa et al., 1998). Dscam is located on chromosome 21q22.2-21q22.3, and shares homolog with genes in the Immunoglobulin(Ig)-superfamily. In mammals and *Drosophila*, Dscam is made up of 24 exons that encode, in order from its N-terminus: a signal peptide, nine Ig superfamily domains, four fibronectin III domains, one more Ig domain, two more fibronectin III domains, a transmembrane domain, and finally a cytoplasmic domain. In addition to Dscam, one other Dscam gene is found in humans. Two Dscam homologs are found in mice, while four have been

identified in *Drosophila melanogaster* (Schmucker, 2007). Northern blot analysis revealed that Dscam mRNA is expressed in the human fetal brain but not the fetal lung, liver, or kidney. In addition, Dscam continues to be expressed in the adult brain at high levels with little expression elsewhere in the body (Yamakawa et al., 1998). In mice, Dscam mRNA is ubiquitously expressed in embryonic neurons in the developing cortical plate and subplate. Dscam protein expression is dynamically regulated during postnatal development in the mouse cortex, with peak expression on days P7 to P10, a period characterized by robust neurite outgrowth and synaptogenesis (Maynard and Stein, 2012). This expression time-course and pattern positions Dscam to play a role in neural development.

Dscam also has unique molecular properties that make it an extremely interesting player in neural development. When transfected into mouse fibroblasts, human Dscam protein leads to enhanced cell adhesion, suggesting that Dscam mediates homophilic intercellular adhesion (Agarwala et al., 2000). In addition, analysis of *Drosophila* Dscam cDNA reveals incredible sequence diversity that arises through alternative splicing for alternative exons in the three Ig domains as well as the transmembrane domain. Ig2, Ig3, and Ig7 each have 12, 48, and 33 potential alternative sequences, respectively. Furthermore, the transmembrane domain has 2 potential splicing variants. This alternative splicing potentially allows for the generation of 38,016 isoforms. Combining homophilic adhesion with alternative splicing, *in vitro* studies indicate that 95% of Dscam isoforms in *Drosophila* only bind with a matching copy of the same isoform (Wojtowicz et al., 2004; Wojtowicz et al., 2007). Analysis of Dscam mRNA expression in *Drosophila* shows that olfactory, mushroom-body, and photoreceptor neurons each express an extensive yet characteristic complement of Dscam isoforms, with each cell expressing several splice forms (Neves et al., 2004; Zhan et al., 2004). Interestingly, mammalian Dscam does not

encode the same sequence diversity as *Drosophila* Dscam, but several studies suggest that despite this, some core functions may be preserved (Fuerst et al., 2009; Fuerst et al., 2008).

2.2.1 The role of Dscam in neural development

Dscam has been shown to play a role in diverse developmental processes including neurite self-recognition (Hughes et al., 2007; Matthews et al., 2007; Soba et al., 2007), synaptic specificity and axon targeting (Cvetkovska et al., 2013), and presynaptic arbor growth (Kim et al., 2013).

2.2.2 Dscam diversity and neurite self-recognition

Early analysis of Dscam in *Drosophila* revealed that is alternatively spliced extensively, suggesting a role for Dscam in neuronal connectivity and patterning. Indeed, Dscam has since been shown to be required for neuronal self-recognition in both axons and dendrites. Neuronal self-recognition is important for proper axonal and dendritic connectivity. On the dendritic side of the neuron, observation of diverse dendritic arbor morphologies reveals that sister dendrites are spaced, spreading out to cover their receptive fields. In the axon, self-recognition is essential for the ability of a nascent axonal branch to separate from the parent branch. Self-recognition allows the newly formed branch to move away from the parent branch instead of fasciculating with the original branch and continuing to both respond to the same signals and to follow the same trajectory. But what are the repulsive signals that mediate self-avoidance in dendrites and axons?

The initial observation that loss of Dscam in *Drosophila* olfactory receptor neurons (ORNs) led to clumped, fasciculated dendrites and reduced dendritic field size raised the question of whether Dscam might act as a repulsive signal mediating self-recognition in dendrites. Furthermore, analysis of ORNs revealed that overexpression of a single Dscam

isoform in two projection neurons that normally exhibited overlapping dendritic fields led to dendritic field segregation (Zhu et al., 2006). These results led to the proposal that the array of Dscam isoforms displayed on the dendritic surface may provide each neuron with a signal that allows it to distinguish its own dendrites from those of neighboring cells. This function is supported by results gleaned from another *Drosophila* system, the larval dendritic arborization (da) neurons (Hughes et al., 2007; Matthews et al., 2007; Soba et al., 2007). Da neurons are sensory neurons which function in the larval body wall as part of the peripheral nervous system. These neurons fall into four classes, each with characteristic dendrite morphology (Grueber et al., 2002). Neurons of all classes display self-avoidance but normally coexist with the dendrites of neighboring neurons of different types. Loss of Dscam leads to self-avoidance defects in all classes of da neurons, causing self-crossing and bundling of dendrites in single *Dscam* mutant clones. This effect is rescued by the overexpression of a single Dscam isoform (Hughes et al., 2007; Matthews et al., 2007; Soba et al., 2007). Additionally, when a single Dscam isoform is overexpressed in all da neurons, strong dendritic avoidance was observed between classes of da neurons which usually inhabit the same territory. This suggests that a single Dscam isoform is sufficient for self-avoidance, but that Dscam diversity is important to permit the coexistence of overlapping dendritic fields within the same territory (Hughes et al., 2007; Matthews et al., 2007; Soba et al., 2007). A similar role for Dscam has been described in the mammalian retina, where retinal ganglion cells in Dscam mutant mice have fasciculated dendrites in addition to clumped cell bodies (Fuerst et al., 2009). This finding suggests a conserved role for Dscam in dendritic self-avoidance between invertebrates and mammals.

Dscam also mediates self-avoidance in axon development. A central brain structure in *Drosophila*, the mushroom bodies, consists of two lobes which are formed by the stereotyped

bifurcation of thousands of axons into a medial and a lateral axon bundle (Ito et al., 1997). Loss of Dscam in mushroom body axons prevents segregation of axon branches into the medial bundle, such that the two axonal branches will fail to bifurcate and will instead fasciculate and continue along the same trajectory (Wang et al., 2002). This effect can be rescued in a single mushroom body neuron by overexpressing a single Dscam isoform (Zhan et al., 2004). In contrast, reduction of isoform diversity to a single Dscam isoform in all mushroom body neurons leads to the formation of a mushroom body that only contains the lateral lobe, a phenotype that is indistinguishable from Dscam loss-of-function (Hattori et al., 2007). In addition, reduction Dscam diversity from 38,016 possible isoforms to 4,752 isoforms led to mushroom bodies that were indistinguishable from those that expressed the full complement of axons (Hattori et al., 2009). These results suggest that it is important that neighboring mushroom body neurons express different Dscam isoforms but that the specific isoforms expressed are unimportant. The vast number of Dscam isoforms may function only to ensure that neighboring neurons express different isoforms, allowing for robust discrimination between self and other.

2.2.3 Dscam's role in synaptic specificity and axon targeting

One of the first specific roles for Dscam in *Drosophila* neural development to be described is that of Dscam in the pathfinding of Bolwig's nerve. Loss of Dscam in this system leads to complete nerve mistargeting in 50% of embryos (Schmucker et al., 2000), suggesting that Dscam also contributes to proper nervous system development by facilitating the targeting for axons to their correct synaptic partners. Indeed, this function is also seen in olfactory neurons, where Dscam is required for axonal targeting to the correct glomerulus in the antennal lobe, such that loss of Dscam leads defined olfactory neuron types to innervate the wrong glomerulus(Hummel et al., 2003). Similarly, Dscam in adult mechanosensory neurons

contributes to the establishment of proper axonal connectivity, and loss of Dscam prevents directed axon extension toward synaptic targets (Chen et al., 2006). The requirements for Dscam diversity in synaptic specificity are different from those of self-recognition. While self-recognition only requires that neighboring neurons express different isoforms without the requirement of specific isoforms, synaptic specificity seems to require not only isoform diversity but also specific isoforms. In both olfactory neurons and mechanosensory neurons, Dscam isoform diversity is required, as re-expression of randomly chosen Dscam isoform does not rescue axonal targeting. In addition, reducing Dscam molecular diversity to 22,176 isoforms also led to defects in mechanosensory neuron targeting, suggesting either that a large number of alternative isoforms are required or that specific isoforms perform functions that are not interchangeable (Chen et al., 2006). Dscam has also been shown to regulate postsynaptic specificity at multiple-contact synapses in the *Drosophila* visual system, where photoreceptor highly-stereotyped tetrad synapses. Loss of Dscam in this system leads to incorrect pairing of synaptic elements, suggesting that Dscam is responsible for excluding inappropriate partners in multiple-contact synapses (Millard et al., 2010). Taken together, these studies suggest that Dscam is important for synaptic specificity and that this role may require not only extensive Dscam isoform diversity but perhaps also defined subsets of Dscam isoforms. However, future research is required to determine whether specific Dscam isoforms are required for proper synaptic targeting in distinct neurons.

2.2.4 Dscam's role in axonal terminal growth

Dscam is also required for proper axon terminal arbor growth. In *Drosophila* C4da neuron axon terminal arbors, loss of Dscam leads to axon terminal arbor truncation, while Dscam overexpression leads to increased axon terminal arbor length (Kim et al., 2013). Furthermore,

fold change in Dscam expression level shows a nearly linear correlation with fold change in presynaptic arbor size. These results suggest that Dscam expression level instructs axon terminal arbor length. Unlike Dscam's roles in self-avoidance and synaptic specificity however, the instruction of presynaptic arbor size by Dscam appears to be isoform diversity-independent. In support of this idea, single C4da neuron that express only a single Dscam isoform (though at the same level as wildtype Dscam) have defects in presynaptic arbor patterning but normal presynaptic arbor length (Kim et al., 2013). Furthermore, randomly selected Dscam isoforms are equally capable of promoting presynaptic arbor growth when overexpressed in C4da neurons. These results suggest a model wherein Dscam's roles in synaptic targeting and presynaptic arbor size control are separable, with the former controlled by isoform diversity and the later controlled by Dscam expression level. Two convergent mechanisms control Dscam expression at the level of protein translation: one involving the dual leucine zipper kinase Wnd that requires the 3' untranslated region of Dscam (Kim et al., 2013) and another involving FMRP (Cvetkovska et al., 2013) that acts on the Dscam coding region (Kim et al., 2013). These two mechanisms work in opposite directions, such increased Wnd expression leads to increased Dscam expression while loss of FMRP leads to increased Dscam expression. Increased Dscam expression in FMRP mutants suggests that Dscam expression level may be higher in Fragile X syndrome, as it is in Down syndrome.

2.3 Materials and Methods

2.3.1 Fly strains

abl^{1} (Gertler et al., 1989), *abl*^{4} (Bennett and Hoffmann, 1992) ppk-Gal4 (Kuo et al., 2005), UAS-Dscam[3.36.25.2]::GFP (Yu et al., 2009), UAS-Abl, UAS-BCR-Abl, UAS-Abl-K417N (Wills et al., 1999), and *dFMRP*$^{\Delta 50M}$ (Zhang et al., 2001) were used in this study.

2.3.2 DNA constructs and generation of transgenic flies

To generate pUASTattB-Abl::Myc for expression in S2 cells, the coding region of Abl was recovered from UAS-Abl transgenic flies by PCR, subcloned into pUASTattB-Myc by using the InFusion cloning system following manufacturer's protocol (Clontech, Mountain View, California). We generated pUASTattB-Abl-K417N::Myc by PCR mutagenesis as previously described (O'Donnell and Bashaw, 2013) from pUASTattB-Abl::Myc. UAS-Dscam[3.36.25.2]::GFP was previously generated as described (Kim et al., 2013). To generate UAS-DscamΔCyto, the Dscam coding region was digested with SstI and ligated with the GFP cDNA.

To generate UAS-Pickles2.31, the Pickles2.31 coding region was subcloned from pCAGGS-Pickles2.31 into pUASTattB using the InFusion cloning system following the manufacturer's protocol (Clontech). Transgenic flies carrying UAS-DscamΔCyto, UAS-Abl::Myc, and UAS-Pickles2.31 were generated by germline transformation with support from BestGene, Inc. Pickles2.31 was generously provided by Dr. Yusuke Ohba at RIKEN Brain Science Institute (Mizutani et al., 2010).

2.3.3 Labeling presynaptic terminals using MARCM

The MARCM technique was used to visualize single neurons homozygous for abl^{1}, abl^{4}, or $dFMRP^{\Delta 50}$, and overexpressing Dscam[3.36.25.2]::GFP as previously described (Kim et al., 2013).

2.3.4 Immunostaining and imaging

Immunostaining of third-instar larvae was accomplished as previously described (Ye et al., 2011). Antibodies used include chicken anti-GFP (Aves, Tigard, Oregon) and rabbit anti-RFP (Rockland, Limerick, Pennsylvania). Samples were dehydrated and mounted with DPX

mounting media (Electron Microscopy Sciences, Hatfield, Pennsylvania). Confocal imaging was completed with a Leica SP5 confocal system equipped with a resonant scanner and 63× oil-immersion lens (NA = 1.40). Images were collected and quantified as previously described (Kim et al., 2013).

2.3.5 S2 cell culture and transfection

Drosophila S2 cells were maintained in Drosophila Schneider's medium supplemented with 10% fetal bovine serum at 25˚C in a humidified chamber. Cells were transfected with indicated DNA constructs together with tubulin-Gal4 (Lee and Luo, 2001) by using Lipofectamine 2000 (Life Technologies, Grand Island, New York) according to manufacturer's protocol.

2.3.6 Co-immunoprecipitation and Western blotting

To perform co-immunoprecipitation, transfected S2 cells were harvested and lysed on ice with lysis buffer (50 mM Tris-HCl/pH 7.4, 150 mM NaCl, 2 mM sodium vanadate, 10 mM sodium fluoride, 1% Triton X-100, 10% glycerol, 10 mM imidazole and 0.5 mM phenylmethylsulfonyl fluoride). Lysates were centrifuged for 15 min at 20,000×g, 4˚C and the resulting supernatant was incubated with Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology, Paso Robles, California) conjugated to mouse monoclonal anti-GFP clone 20 (Sigma-Aldrich, St. Louis, Missouri) for 4 hr at 4˚C. After washing once with lysis buffer, twice with lysis buffer containing 0.1% deoxycholate, and 3 times with lysis buffer lacking Triton X-100, the immunoprecipitates and total lysates were resolved on 7.5% SDS-PAGE gels followed by western blot analysis as previously described (Kim et al., 2013). Primary antibodies used in western blotting were mouse monoclonal anti-tubulin (Sigma), mouse anti-Myc (Sigma-Aldrich), mouse monoclonal anti-Aequorea Victoria GFP JL-8 (Clontech), and rabbit anti-phospho-Tyr412-c-Abl (Cell Signaling, Beverly, Massachusetts).

2.3.7 In vivo Abl activity assay with Pickles2.31

To assay in vivo Abl activation, UAS-Pickles2.31 was expressed specifically in C4da neurons using ppkGal4 along with other UAS transgenes. The CNS was dissected from third-instar larvae into ice-cold PBS with 2 mM sodium vanadate (~100 per experimental condition). After a brief centrifugation, larval CNSs were transferred into lysis buffer as described above in immunoprecipitation and western blotting. Cells were disrupted using a pestle followed by brief sonication. Immunoprecipitation and western blotting of Pickles2.31 was then accomplished as described above. Primary antibodies used were rabbit anti-eGFP (a gift from Dr Yang Hong) and rabbit anti-phospho-Tyr 207-CrkL (Cell Signaling).

2.3.8 Drug treatment of Drosophila larvae and S2 cells

Nilotinib (Abcam, United Kingdom) and bafetinib (ApexBio Technology, Houston, Texas) were dissolved in dimethyl sulfoxide (DMSO) at 94 mM and 50 mM, respectively, as stock solutions before adding to S2 cells or fly food. S2 cells transfected with Abl::Myc were treated with either 5 μM nilotinib or the same volume of DMSO as a vehicle control for 6 hr before harvested and subjected to western blot analysis. Nilotinib and bafetinib were administered to larvae by rearing the larvae on standard corn meal food containing different concentrations of the drugs. The highest concentrations that did not affect overall larval development were used. Fly viability on nilotinib treatment was performed by counting the number of adult flies. Seven virgin female and seven male flies were crossed and transferred to standard corn meal food containing either 380 μM nilotinib or the same volume of DMSO (0.4% final concentration). Embryos were collected for 24 hr and allowed to develop. Eclosed adult flies were counted on a daily basis. The MARCM technique was used to generate and visualize mutant single C4da neurons as described above except that Drosophila embryos were collected and raised for 4 days on standard corn

meal food containing either 380 μM nilotinib, 125 μM Bafetinib, or 0.4% DMSO. Sample preparation, imaging, and quantification were then completed as described above.

2.3.9 Colocalization analysis

Colocalization of Dscam and Abl was quantified with Manders' Correlation Coefficients using the Just Another Colocalization Plugin (JACoP) (Bolte and Cordelieres, 2006) in ImageJ. Images were analyzed in three dimensions. Manders' Correlation Coefficients vary between 0 and 1, with 0 representing no overlap between images and 1 representing complete colocalization. M1 and M2 describe the overlap of each channel with the other (Bolte and Cordelieres, 2006). M1 presents a measure of the fraction of Abl::Myc that overlaps Dscam(ΔCyto)::GFP, while M2 presents a measure of the fraction of Dscam(ΔCyto)::GFP that overlaps Abl::myc.

2.3.10 Statistical analysis

Two-way student's t- test was used for statistical analysis. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$; ns: not significant.

2.4 Results

2.4.1 Abl is sufficient to promote presynaptic arbor growth in C4da neurons

To delineate the molecular mechanisms of Dscam signaling in axon terminal arbor development we took advantage of the *Drosophila* C4da neuron system, which has previously been used to study the effects of Dscam expression level during development (Hughes et al., 2007; Kim et al., 2013; Matthews et al., 2007; Soba et al., 2007). From tests of candidate genes that potentially mediate Dscam function, including focal adhesion kinase, proto-oncogene tyrosine-protein kinase Fyn, p21 Activated Kinase, Ras homolog gene family member A, and Abelson tyrosine kinase (Abl), we identified Abl as a key molecule mediating Dscam's functions

in presynaptic terminal growth. I first asked whether Abl is sufficient to promote presynaptic terminal arbor growth in C4da neurons. Consistent with a previous study performed in *Drosophila* adult CNS neurons (Leyssen et al., 2005), overexpression of Abl in C4da neurons caused significant overgrowth of the presynaptic terminals (Figure 2.1 a,b,e). Since Abl is known to have both kinase-dependent and kinase-independent functions (Henkemeyer et al., 1990; Schwartzberg et al., 1991; Tybulewicz et al., 1991), I tested whether expression of a kinase-dead form of Abl, Abl-K417N (Henkemeyer et al., 1990; Wills et al., 1999), could promote presynaptic terminal growth. I found that C4da presynaptic terminals overexpressing Abl-K417N were indistinguishable from wild-type (Figure 2.1 d,e), indicating that Abl kinase activity is required. Based on this result, I hypothesized that if presynaptic terminal growth is dependent on Abl kinase activation, overexpression of a constitutively active form of Abl should lead a greater increase in presynaptic terminal growth than Abl alone. One well-known constitutively active form of Abl is BCR-Abl, which results from reciprocal translocation between chromosomes 9 and 22 in human patients with chronic myeloid leukemia. This translocation leads to the juxtaposition of the *Abl* gene with the *Breakpoint Cluster Region* (*BCR*) gene, resulting in a fusion protein that has constitutive Abl kinase activity (Rowley, 1973). Consistent with the idea that Abl kinase activation is important, overexpression of constitutively active BCR-Abl led to extremely exuberant presynaptic terminal overgrowth (Figure 2.1 c,e). Taken together, these results suggest that Abl is sufficient to promote presynaptic terminal growth and that the extent to which Abl instructs presynaptic terminal growth is related to the level of Abl kinase activation.

2.4.2 Dscam requires Abl to promote presynaptic terminal growth

Since overexpression of Abl increases presynaptic terminal growth, similar to Dscam, I next tested whether Dscam requires Abl to instruct presynaptic terminal growth. For this, I used the mosaic analysis with a repressible cell marker (MARCM) technique to overexpress Dscam in abl^{1} mutant C4da neurons (Lee and Luo, 2001) and assessed presynaptic terminal length. Importantly, I was able to investigate the cell-autonomous consequences of both Dscam overexpression and *abl* loss-of-function using this technique. I found that although Dscam overexpression led to significantly (150%) longer presynaptic terminals than wildtype clones (Figure 2.1 f,g,o), abl^{1} mutant clones that overexpressed Dscam did not differ in length from abl^{1} mutant clones (Figure 2.1 h,i,o). Presynaptic terminal length was also subtly but significantly shorter in abl^{1} mutant clones compared to wild-type controls (Figure 2.1 i,o). A different loss-of-function allele of *abl*, abl^{4}, exhibited similar effects on the presynaptic overgrowth caused by Dscam overexpression (Figure 2.1 j,k,o), confirming that loss of *abl* function is responsible for blocking the presynaptic phenotypes caused by increased Dscam levels. Consistent with the idea that Abl is not involved in trafficking Dscam to the presynaptic arbors, *abl* loss-of-function mutations did not affect the expression of the Dscam transgenes in the C4da cell body or presynaptic terminals (Figure 2.2).

As a further proof-of-concept, I asked whether loss of *abl* could mitigate the effects of dysregulated Dscam levels without utilizing Dscam transgenes. Fragile X syndrome is caused by an absence of FMRP (Kremer et al., 1991), and is modeled in Drosophila using loss-of-function mutants for the Drosophila homolog of *FMR1*, *dFMRP* (Dockendorff et al., 2002; Zhang et al., 2001). It has previously been shown that FMRP binds to Dscam mRNA in both mammals and *Drosophila* (Cvetkovska et al., 2013; Darnell et al., 2011; Kim et al., 2013) and that *dFMRP*

represses Dscam expression to control presynaptic terminal growth, so that *dFMRP* mutants exhibit increased presynaptic terminal length in C4da neurons (Kim et al., 2013). Strikingly, loss of only a single copy of *abl* significantly rescued presynaptic terminal length to wild-type levels (Figure 2.1 l–n,p). These results suggest that Abl is required for Dscam to instruct presynaptic terminal growth.

2.4.3 Abl is not required for Dscam's role in dendritic self-avoidance

An important function of Dscam in neuronal development is mediating self-avoidance between neurites of the same neuron (Zipursky and Grueber, 2013). Abl does not seem to be required by Dscam for either dendrite growth, as loss of Abl did not significantly alter dendritic length in C4da neurons, or for dendritic self-avoidance in C4da neurons, as no dendritic fasciculation or clumping was observed in *abl* mutant neurons (Figure 2.3). To further test whether *abl* is required for dendritic self-avoidance, I used a previously described ectopic dendritic avoidance assay. In the *Drosophila* larval peripheral nervous system, the dendrites of dendritic arborization (da) neurons project across the body wall and can be placed into four morphologically-distinct classes, I-IV. While sister dendrites of all da neurons show self-avoidance, the dendrites of neighboring neurons of different classes overlap extensively (Grueber et al., 2002). Previous studies have shown that all classes of da neurons require Dscam for self-avoidance. Overexpression of a single Dscam isoform in two neurons whose dendritic fields normally overlap, class I neuron vpda and class III neuron v'pda, causes their dendritic fields to segregate (Hattori et al., 2007; Hughes et al., 2007; Matthews et al., 2007). These results suggest that Dscam is both necessary and sufficient to cause dendritic self-avoidance in da neurons. Consistent with previous reports, I found that the dendritic territories of wildtype class I vpda and class III v'pda neurons overlapped extensively, while overexpression of a single form of

Dscam caused their dendritic territories to segregate as assessed by counting dendritic crossings of class I and class III dendrites (Figure 2.4 a,b,d). In addition, I found that loss of *abl* did not compromise the ectopic avoidance caused by overexpressing Dscam in these distinct types of da neurons (Figure 2.4 c,d). This suggests a divergence in Dscam signaling between presynaptic terminal and dendritic branch development. Taken together, these results indicate that Abl is specifically required for Dscam-mediated presynaptic terminal growth.

2.4.4 Dscam and Abl bind through the Dscam cytoplasmic domain in culture and in vivo

Next, I asked how Abl might mediate Dscam signaling. Abl is a cytoplasmic tyrosine kinase with high sequence conservation between Drosophila and mammals. Abl contains an SH3 domain, SH2 domain, and a kinase domain. In its inactivate state, these SH3 and SH2 domains bind in a closed conformation to an SH3-binding site in the Abl linker domain and to the Abl kinase domain, respectively. However, intercellular binding to SH3- and SH2-binding sites on other molecules, including the cytoplasmic domain of membrane receptors, moves Abl into an active conformation, which in turn leads to catalytic activation of Abl (Bradley and Koleske, 2009). To test whether Abl might be binding to the cytoplasmic domain of Dscam, we created a Dscam transgene that lacks most of the cytoplasmic domain, deleting the last 877 base pairs of the intracellular domain including half of exon 18 and all of exons 19 through 23 (DscamΔCyto). In contrast to the exuberant presynaptic terminal overgrowth caused by Dscam overexpression in C4da neurons (Figure 2.5 a, middle), overexpressing DscamΔCyto did not cause presynaptic terminal overgrowth (Figure 2.5 a, bottom). DscamΔCyto was trafficked to the axon terminals and expressed at a similar level to full length Dscam (Figure 2.6). These results suggest that the cytoplasmic domain is required for Dscam to instruct presynaptic terminal growth. I then asked whether Dscam and Abl physically interact with each other through the Dscam cytoplasmic

domain. I found that Dscam and Abl proteins co-immunoprecipitated from transfected *Drosophila* Schneider 2 (S2) cells expressing these two proteins (Figure 2.5 b, second lane from right). In contrast, DscamΔCyto did not co-immunoprecipitate Abl (Figure 2.5 b, furthest right lane). These results suggest that Dscam and Abl proteins form a complex through Dscam's cytoplasmic domain.

Next, to test the in vivo interaction of Dscam and Abl in presynaptic terminals specifically, I determined whether Abl localization in presynaptic terminals was altered by the expression of Dscam or DscamΔCyto (Figure 2.5 c). When expressed alone or with DscamΔCyto::GFP, Abl::Myc was diffusely distributed in the presynaptic terminals, showing scant colocalization with DscamΔCyto::GFP (Figure 2.5 c, middle and bottom). However, when expressed with Dscam::GFP, Abl::Myc became more punctate and clearly colocalized with Dscam::GFP (Figure 2.5 c, top). I used Manders' Correlation Coefficients to quantify the colocalization of Dscam::GFP and Abl::Myc. Colocalization analysis revealed a significant increase in both M1 and M2 (Figure 2.5 c, bottom right) when Abl::Myc was coexpressed with Dscam::GFP as compared to when Abl::Myc was coexpressed with DscamΔCyto::GFP, where M1 represents the fraction of Abl that overlaps with Dscam, and M2 represents the fraction of Dscam that overlaps with Abl. These findings support the idea that Abl and Dscam interact in presynaptic terminals in vivo.

2.4.5 Dscam activates Abl in culture and in C4da presynaptic arbors in vivo

Do increased Dscam levels activate Abl kinase? In mammals, autophosphorylation of Abl at tyrosines 245 and 412 (Y245 and Y412) stabilizes the active conformation of the kinase (Brasher and Van Etten, 2000; Tanis et al., 2003). As a result, phospho-specific antibodies raised against Y412 have been employed to detect active Abl kinases (Brasher and Van Etten, 2000).

Since Abl is highly conserved between *Drosophila* and mammals, this approach has been used successfully to recognize the phosphorylation of the corresponding tyrosines (Y539/522) in Drosophila as an assay for Abl kinase activation (Stevens et al., 2008). Since the ability of Abl to instruct presynaptic terminal growth relies on Abl kinase activity, I tested whether Dscam activates Abl using a phosho-Y412-Abl (p-Abl) antibody. I found that Abl kinase activation was significantly increased (2.6 fold) when Abl and Dscam were co-expressed in S2 cells (Figure 2.7 a). Furthermore, unlike wild-type Dscam, DscamΔCyto did not increase Abl kinase activation. In fact, it appears to act as a dominant-negative, as Abl activity was significantly decreased from control (Figure 2.7 a, right). As a negative control, no signal was detected when the kinase-dead Abl-K417N was blotted with p-Abl antibody in the same assay, suggesting that our assay specifically reported Abl activation (Figure 2.8). These results suggest that Dscam enhances Abl kinase activity.

To investigate whether the same is true in presynaptic terminals in vivo, I devised a novel method of reporting Abl activation specifically in C4da presynaptic terminals. To achieve this, I used a previously described probe that reports Abl activity, Phosphorylation indicator of CrkL en substrate number 2.31 (Pickles2.31) (Mizutani et al., 2010). Designed as a Förster resonance energy transfer (FRET) probe, Pickles2.31 is composed of a fragment of a characteristic Abl substrate, CrkL, sandwiched between the fluorescent proteins Venus and enhanced CFP (ECFP) (Figure 2.7 b). It has previously been reported that activated Abl phosphorylates Pickles2.31 on the Y207 residue of the CrkL fragment, which can be detected with an antibody against CrkL-phospho-Y207 (p-CrkL) (Mizutani et al., 2010). After expressing Pickles2.31 specifically in C4da neurons with the ppk-Gal4 driver, I dissected the larval CNS and immunoprecipitated Pickles2.31 from the lysates. Since the cell bodies of C4da neurons reside in the body wall, using

only the larval CNS allowed us to monitor Pickles2.31 phosphorylation only in the C4da neuron presynaptic terminals (Figure 2.7 c). Consistent with the notion that Pickles2.31 is an Abl activity indicator, overexpression of BCR-Abl led to a robust increase in phospho-Y207 levels as compared to the control. Furthermore, I found that overexpression of Dscam in C4da neurons led to an increase in Y207 phosphorylation of Pickles2.31 in the presynaptic terminals, while overexpression of DscamΔCyto was indistinguishable from control (mCD8-mRFP) (Figure 2.7 d). These results suggest that Dscam activates Abl both in culture and in C4da presynaptic terminals in vivo, and that this activation requires the cytoplasmic domain of Dscam.

2.4.6 Inhibition of Abl with tyrosine kinase inhibitors mitigates the consequences of increased Dscam expression in C4da presynaptic arbors

These results raised the interesting possibility that targeting Abl might be a viable therapy for brain disorders caused by increased Dscam expression. Abl is a well-established target for treating chronic myeloid leukemia, and there are multiple Abl inhibitors that are approved by the US Food and Drug Administration (FDA). As a proof-of-concept experiment, we attempted to rescue the developmental defects caused by Dscam overexpression using Abl inhibitors. We first tested nilotinib, which is an FDA-approved second-generation Abl kinase inhibitor that can cross the blood–brain barrier (Weisberg et al., 2005) (Hebron et al., 2013). Using cultured S2 cells overexpressing Abl, we found that nilotinib robustly inhibited Drosophila Abl (Figure 2.9 a). Based on these results, we tested whether administration of nilotinib to developing larvae could rescue the effects of increased Dscam expression in C4da presynaptic terminals in vivo. To do this, we performed MARCM to visualize single C4da neurons in animals fed nilotinib or vehicle and assessed presynaptic terminal length. While overexpression of Dscam caused increased (152%) presynaptic terminal length in animals fed vehicle (Figure 2.9 b–d), the effect was

significantly rescued (to 115% of control) by feeding the animals with nilotinib (Figure 2.9 b,e). Consistent with the idea that these effects were due to inhibition of Abl activity rather than a reduction in Dscam expression, nilotinib did not change the expression of the Dscam-GFP transgene (Figure 2.10). Administration of nilotinib to developing larvae did not adversely affect overall development and neuronal growth. At the dose we used, nilotinib did not cause changes in presynaptic terminal growth (Figure 2.9 f) or dendritic growth (Figure 2.11 a,b) in wild-type larvae. Moreover, it did not impact the number of adults that eclosed or the dynamics of eclosion when compared to vehicle-fed flies (Figure 2.11 c,d). Although frequently used to inhibit pathological increases in Abl activity in patients, nilotinib is known to have several off-targets, including c-Kit, PDGFR, Arg, NQ02, and DDR1 (Hantschel et al., 2008). Consistent with the idea that nilotinib acts on Abl rather than on an off-target molecule to rescue presynaptic terminal growth, administering nilotinib to larvae overexpressing Dscam in abl^{1} mutant single C4da neurons did not lead to a further decrease in presynaptic terminal length when compared to vehicle-fed control (Figure 2.21 c,d). To further rule out the possibility that the observed rescue of presynaptic terminal length by nilotinib was the result of an off-target effect, we tested bafetinib, another Abl inhibitor with non-overlapping off-targets, Fyn and Lyn (Kimura et al., 2005). Bafetinib has also been shown to cross the blood-brain barrier (Santos et al., 2010). Like nilotinib, administration of bafetinib to Dscam-overexpressing larvae led to a significant decrease in presynaptic terminal length (Figure 2.12 a,b,f,g) without changing the expression of the Dscam transgene (Figure 2.10 b). Bafetinib alone did not change presynaptic terminal length in wild-type larvae when compared to wild-type larvae fed vehicle (Figure 2.12 egG). Taken together, these results suggest that pharmacological inhibition of Abl mitigates the consequences of increased Dscam signaling in vivo. We next sought to test the efficacy of nilotinib treatment

in a model of a disease associated with dysregulated Dscam expression, Fragile X syndrome. Thus, we tested whether administration of nilotinib could rescue the presynaptic overgrowth caused by increased Dscam expression in *dFMRP* mutants. We found that, while *dFMRP* mutants fed vehicle showed a significant increase (130%) in presynaptic terminal length (Figure 2.9 b,g), administration of nilotinib to *dFMRP* mutants almost completely rescued (to 103% of control) the exuberant presynaptic terminal growth to wild-type levels (Figure 2.9 b,h). These results suggest that pharmacological inhibition of Abl kinase is effective for mitigating the effects of increased Dscam level in an in vivo model of Fragile X syndrome.

2.5 Discussion

In this study, I show that Dscam requires Abl to promote presynaptic terminal growth in vivo and that the binding of Abl to the Dscam cytoplasmic domain leads to Abl kinase activation. Furthermore, we show that treating *Drosophila* larvae with Abl inhibitors rescues the developmental defects caused by increased Dscam levels in vivo in both Dscam-overexpressing neurons and disease-relevant models.

Although Abl has previously been shown to promote axon arborization in *Drosophila* (Leyssen et al., 2005), this was the first study reporting that Abl promotes presynaptic terminal arbor growth in C4da neurons. Interestingly, I found that although overexpression of wildtype Abl increased presynaptic terminal length, this effect was mild. This may suggest that even when Abl expression is increased, Abl activation remains tightly controlled. The overexpression of BCR-Abl led to extremely increased presynaptic terminal growth, to a similar extent to that seen when Dscam is overexpressed in all C4da neurons. Consistent with our findings, this suggests that Dscam may activate Abl, so that only overexpression of activated Abl mirrors the Dscam overexpression phenotype.

In addition, although Dscam and BCR-Abl overexpression lead to exuberant increases in presynaptic arbor growth, the phenotypes of each of these treatments appear subtly different. To the trained eye, it is clear that overexpression of BCR-Abl leads to an increased number of connectives, but that these connectives are still relatively well fasciculated. In contrast, although overexpression of Dscam also leads to an increased number of connectives, the connectives are not well fasciculated and thus spread out to cover a wider space, making each connective appear wider and narrowing the space in the middle of the axon ladder between the contralateral connective. I speculate that this "avoidance" phenotype in Dscam overexpression results from Dscam's known function in neuronal self-avoidance, which has previously been reported in both dendrites and axons. Since a single Dscam isoform was expressed in all C4da neurons, neighboring presynaptic terminals avoid one another, resulting in wider and less fasciculated connectives. I hypothesize that if Dscam expression were increased in C4da neurons without altering the relative levels of each Dscam isoform, the phenotype would closely resemble that of overexpression BCR-Abl.

I show that Dscam requires Abl to instruct presynaptic terminal growth. However, although loss of *Dscam* has been shown to result in severely truncated presynaptic terminals (Kim et al., 2013), loss of *abl* only leads to a subtle, though significant, decrease in presynaptic terminal length. This is counterintuitive because if Dscam's effect in presynaptic terminal growth is mediated entirely through Abl, one would expect *Dscam* and *abl* loss-of-function phenotypes to be identical. This subtle effect of *abl* loss-of-function is likely to be explained by perdurance and maternal contribution. Using the MARCM system, *abl* function will be completely lost in a MARCM clone upon its final division. However, Abl protein present before this division may persist, a phenomenon known as perdurance. Furthermore, *Drosophila* embryos that have no

zygotic *abl* but whose mothers are heterozygous for *abl* mutations live until the late larval stage. In contrast, *Drosophila* embryos that lack both maternal and zygotic *abl* die before the completion of embryogenesis, suggesting that Abl mRNA is maternally contributed (Bennett and Hoffmann, 1992; Grevengoed et al., 2001). Taken together, the subtle effect we see in our abl^{1} and abl^{4} MARCM results are probably due to a combination of both perdurance and maternal contribution. A future experiment that would confirm this hypothesis is to generate *Drosophila* embryos that lack both zygotic and maternal *abl* (Chou and Perrimon, 1996) and then examine C4da presynaptic terminals using ppk-eGFP.

We show here that increased Dscam expression leads to increased Abl activation both in culture and in vivo. The observation that DscamΔCyto may act as a dominant negative is intriguing, as the mechanism by which this would occur in unclear. This suggests the influence of other molecular partners and could be explained if Dscam works in concert with another protein that interacts with Dscam via the Dscam extracellular domain. In this scenario, loss of the Dscam cytoplasmic domain would render Dscam incapable of activating Abl, but would not change its interactions with its other partner through the extracellular domain. Therefore, DscamΔCyto would bind up a majority of the functional partner, leaving the remaining wildtype Dscam with fewer opportunities to interact.

Our in vivo Abl activation assay with Pickles2.31 represents the first time, to our knowledge, that Abl activation has been assayed in a cell-type-specific manner in *Drosophila* in vivo. Moreover, this assay is unique in that it allows assessment of Abl activation in a cell-compartment-specific manner – axon terminals in this case. The success of this assay also demonstrates that mammalian CrkL is phosphorylated by *Drosophila* Abl, further evidence that Abl function is highly conserved between *Drosophila* and mammalian systems. Future studies

could extend this assay to develop an in vivo FRET assay that directly measures FRET efficiency in C4da presynaptic arbors.

Furthermore, in the course of our in vivo Abl activity assay using Pickles2.31, we found that the phospho-CrkL antibody we used also recognizes a phosphorylated tyrosine on the Dscam cytoplasmic domain. Importantly, this site is only phosphorylated when Dscam is co-expressed with Abl, so that no signal is detected at the site of the Dscam band if Dscam is expressed without Abl. This suggests that Abl phosphorylates a site on Dscam. Furthermore, we found that when Abl was co-expressed with a Dscam transgene with all of the cytoplasmic tyrosines mutated to phenylalanine (Dscam-AllF), phospho-Dscam staining was no longer visible, indicating that Abl phosphorylates one or more tyrosines in the Dscam cytoplasmic domain (Figure 2.13). This suggests a mechanism wherein Abl binds to Dscam via an SH3-binding site in the Dscam cytoplasmic domain and then phosphorylates a tyrosine, creating an SH2-binding site. Abl will, in turn, may then bind to the SH2-binding site that has been created, leading to increased Abl activation. Further research is required to test this hypothesis, but detailed knowledge of the sites and mechanisms that allow Dscam to activate Abl may open the door for rationally designed therapeutics that disrupt Dscam-Abl binding.

Finally, our results suggest that Abl inhibition with FDA-approved tyrosine kinase inhibitors can block morphological changes caused by increased Dscam expression. This result is proof of the concept that disrupting Dscam signaling might prove therapeutic for disorders in which Dscam signaling is dysregulated, like Down syndrome or Fragile X syndrome. Our results suggest that the tyrosine kinase inhibitors we tested are acting through inhibition of Abl, as two such drugs with non-overlapping off-targets rescued presynaptic terminal length in *Drosophila* larvae that overexpressed Dscam. Importantly, feeding tyrosine kinase inhibitors to wild-type

flies did not lead to decreases in presynaptic terminal length when compared to flies fed vehicle. This result is significant because it suggests that this drug treatment is targeting pathogenic activation of Abl and does not interfere with development of presynaptic terminals in general. This result may be explained by two different hypotheses. On one hand, tyrosine kinase inhibitors may inhibit some Abl but not all Abl. This means that a low level of Abl activation is maintained in the treated flies, allowing Abl to perform its normal physiological functions. On the other hand, Abl activity may not play an important role in the normal development of presynaptic terminals, so that inhibiting Abl during development would not affect the final length of presynaptic terminals. We favor the former explanation, as the later does not fit with our observation that loss of *abl* leads to significantly shorter presynaptic terminals, nor does it explain the axonal truncation previously reported in *Drosophila* embryos lacking both maternal and zygotic Abl (Grevengoed et al., 2001).

The results of this study present the first signaling mechanism of Dscam in presynaptic terminal growth. It is hoped that these findings will open the door for discovery of more detailed understanding of how changes in Dscam expression level impact presynaptic arbor growth.

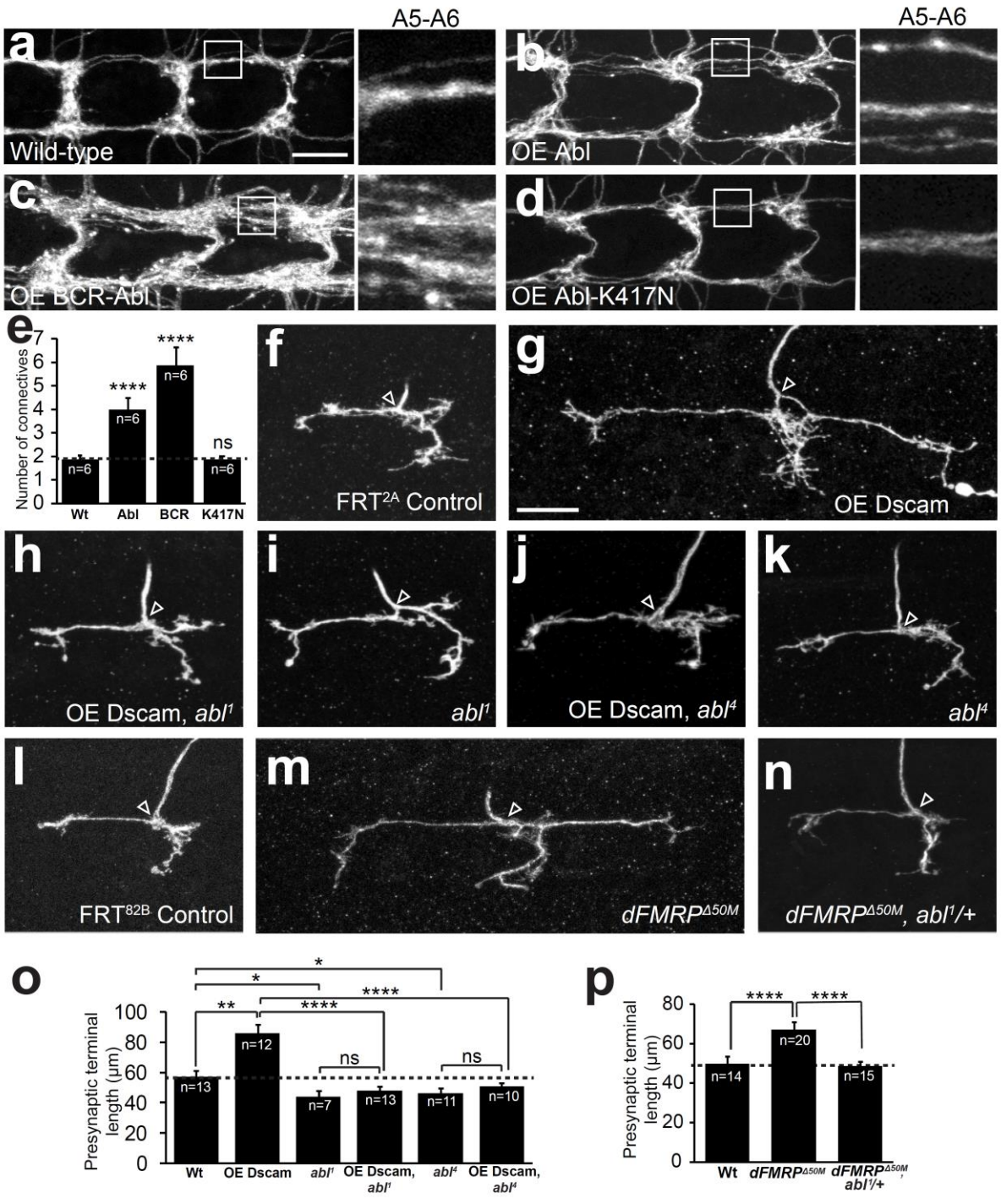

Figure 2.1 Dscam requires Abl to promote presynaptic terminal growth.
(a-d) Abl is sufficient to cause axon terminal overgrowth in C4da neurons. Transgenes were expressed with a C4da neuron-specific Gal4 driver, ppk-Gal4, and presynaptic terminals were visualized with membrane monomeric RFP (mRFP) transgene. Overexpression of Abl (b) leads

to a modest increase in presynaptic terminal growth when compared to control (a). Overexpression of constitutively active BCR-Abl (d) leads to extremely robust increased presynaptic terminal growth, while overexpression of kinase-dead Abl-K417N (c) is indistinguishable from control. Scale bars are 5 μm. (e-j) Abl is required in C4da neurons for Dscam to instruct presynaptic terminal growth. The MARCM technique was used to generate and visualize single mutant C4da neurons. While overexpression of Dscam::GFP (f) in single C4da presynaptic terminals leads to increased length when compared to control (e), overexpression of Dscam in abl1 mutant neurons leads to presynaptic terminal lengths that are indistinguishable from abl1 mutant neurons (g). Similarly, overexpression of Dscam in abl4 mutant neurons (j) does not significantly change presynaptic terminal length when compared to abl4 mutant neurons (i). Scale bar is 10 μm. (k) Quantification of the presynaptic arbor length in C4da neurons of indicated genotypes. Sample number is shown in white within each bar.

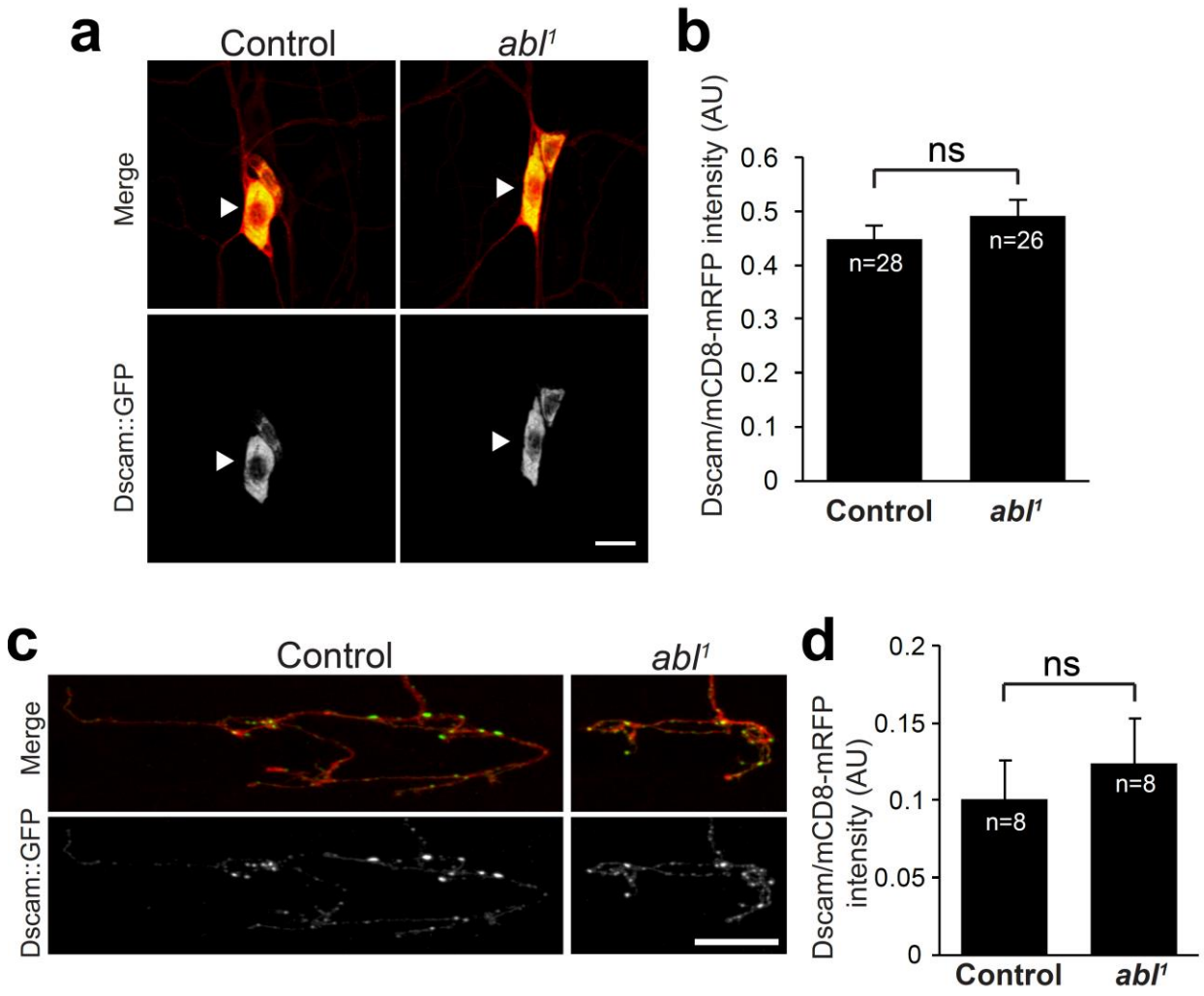

Figure 2.2 Loss of Abl does not affect Dscam-GFP expression level.
(a) Example images of C4da neuron cell bodies (white arrowheads) in control (left) or *abl*^{1} homozygous mutant (right) animals. Upper images show merged signals of cCD8::mRFP and Dscam::GFP, while lower images show Dscam-GFP alone. Scale bar is 5 µm. (b) Quantification of the relative intensity of Dscam::GFP fluorescence normalized to CD8-mRFP. Sample number is shown inside each bar.

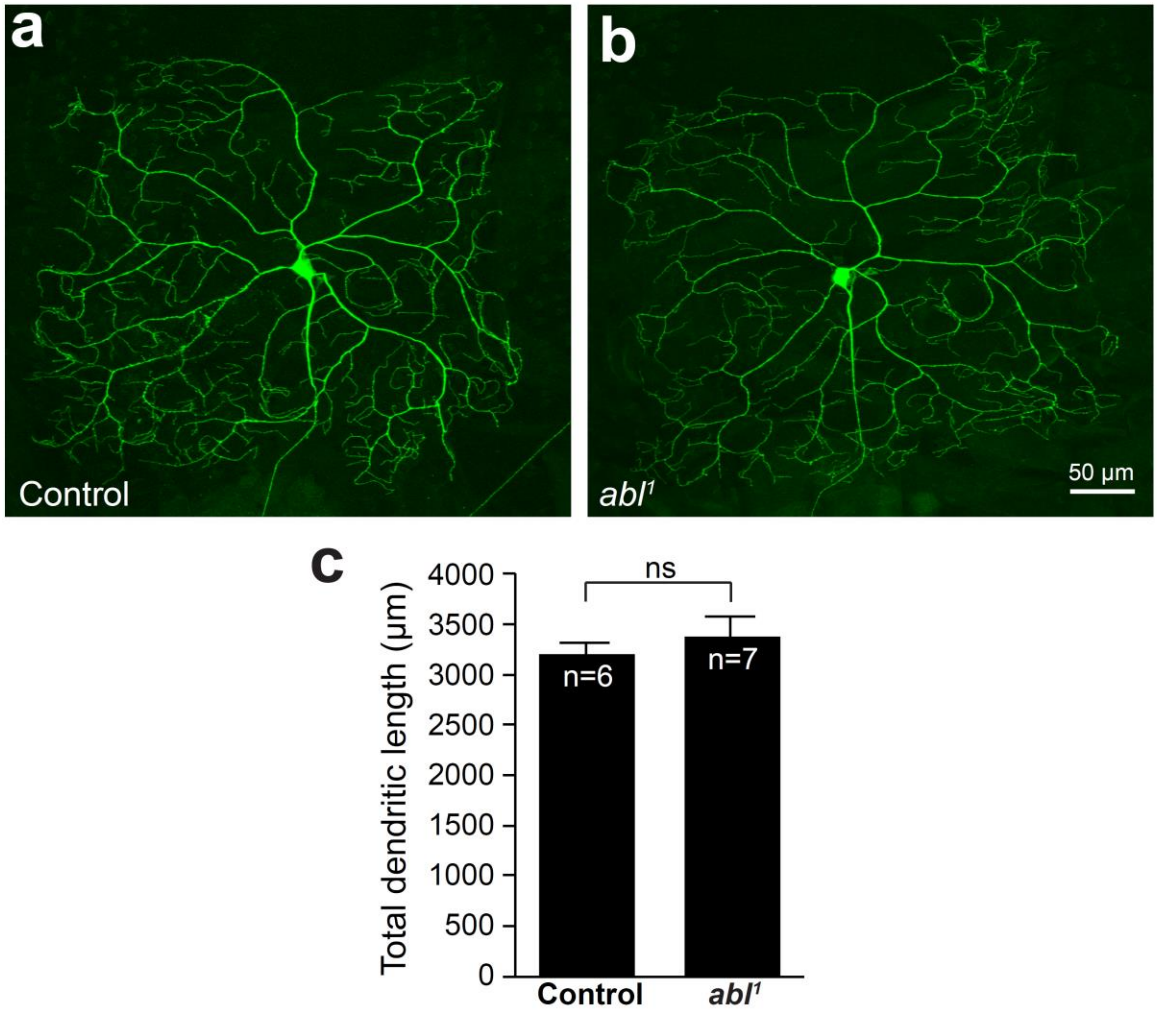

Figure 2.3 Loss of abl does not affect C4da dendritic length or morphology.
Representative images of control (a) and abl1 mutant C4da neuron clones (b). The average total dendritic length is not significantly different between these two conditions (c). Scale bar is 50 µm.

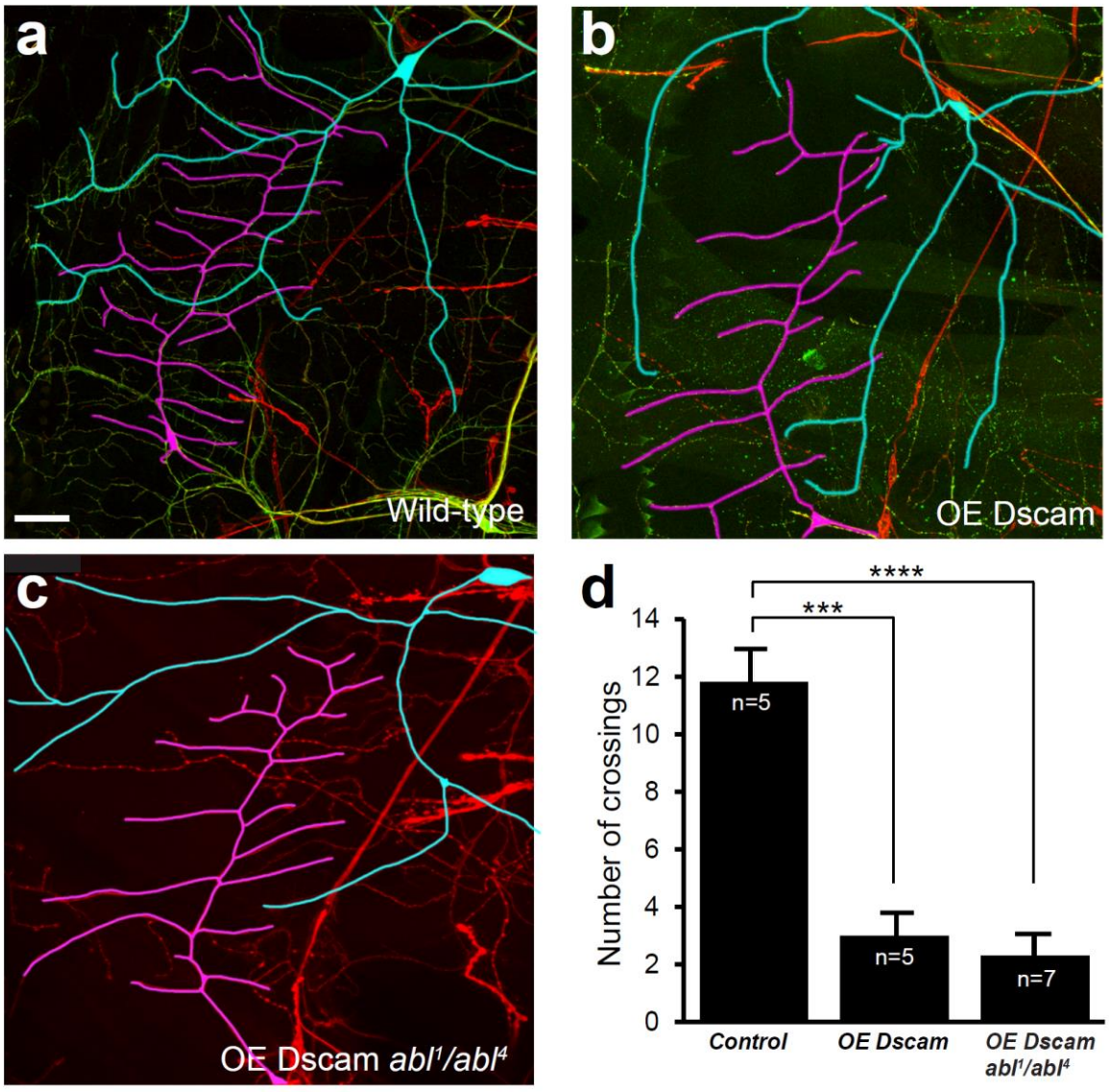

Figure 2.4 The single Dscam isoform-induced ectopic repulsion between class I and class III dendrites does not require *Abl*.
The dendritic field of the class I da neuron vpda (traced in magenta) normally overlaps extensively with that of the class III da neuron v'pda (traced in cyan) (a). When a transgene expressing a single Dscam isoform is overexpressed in both neurons, their dendritic fields segregate (b), exhibiting an ectopic repulsion. The expression of the same Dscam transgene in *abl*^{1} neurons also leads to ectopic repulsion. Original background images show the pan-neuronal marker labeled with anti-Horseradish-peroxidase antibody (red) and Dscam::GFP transgene expression (green). (d) Quantification of the number of dendritic branch crossing. Sample number is shown in white inside each bar. Scale bar is 25 μm.

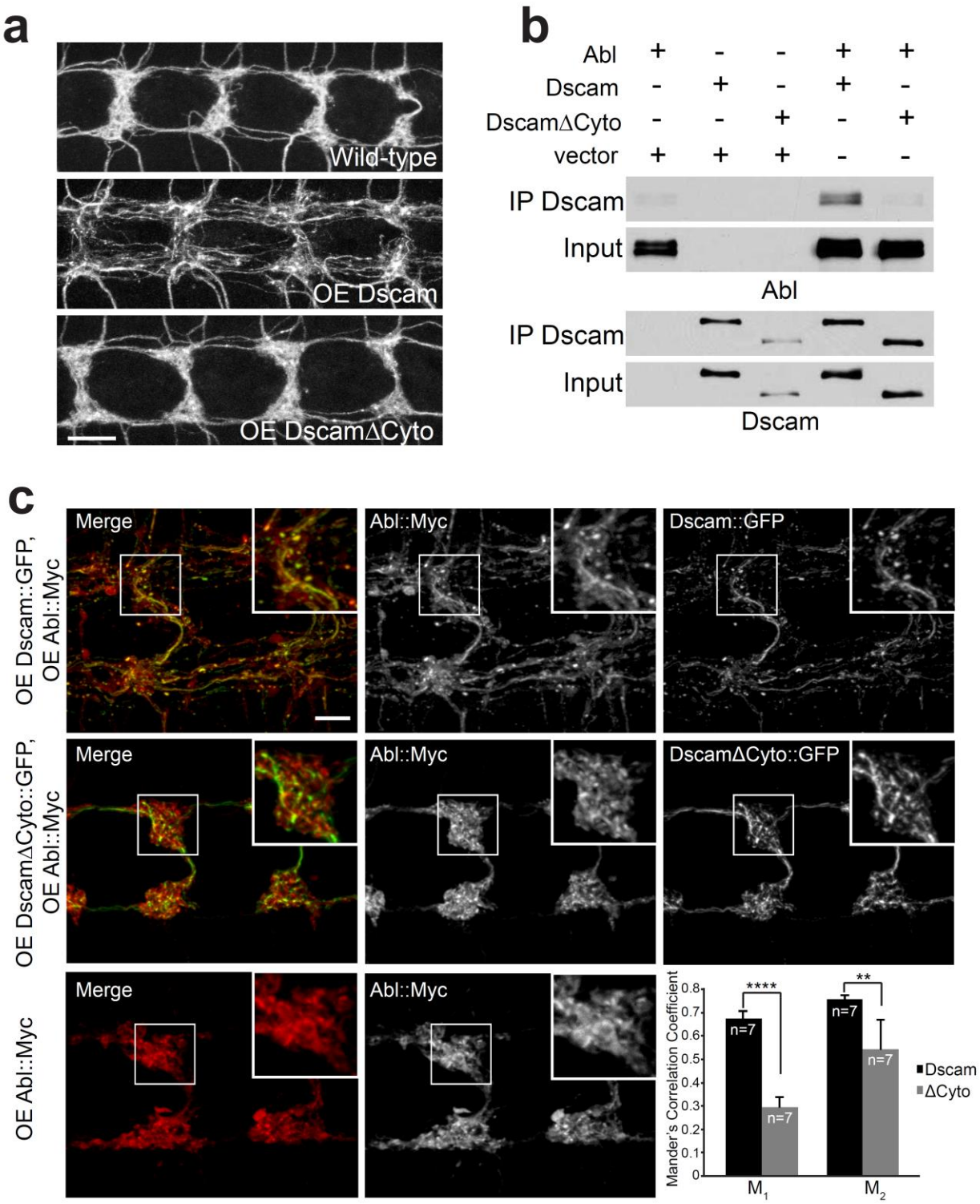

Figure 2.5 Dscam binds to Abl through its cytoplasmic domain.
(a) The cytoplasmic domain of Dscam is required for instructing presynaptic terminal growth. Overexpression of full-length Dscam under the control of ppk-Gal4 (a, middle) leads to exuberant presynaptic terminal overgrowth when compared to control (a, top). However, overexpression of DscamΔCyto a, bottom) fails to increase presynaptic terminal growth. Scale

bar is 10 μm. (b) Dscam binds Abl via its cytoplasmic domain. S2 cells were co-transfected with Abl::Myc along with either Dscam::GFP, DscamΔCyto::GFP, or an empty vector. Dscam::GFP was immunoprecipitated with anti-GFP antibody and bound Abl::Myc was examined with anti-Myc antibody (top). Immunoprecipitated Dscam::GFP and input Dscam::GFP was examined with anti-GFP (bottom). (c) Abl colocalizes and redistributes with Dscam but not with DscamΔCyto in presynaptic terminals in vivo. When expressed alone, Abl::Myc shows a diffuse pattern (bottom). When expressed along with Dscam::GFP (top), Abl::Myc redistributes into punctate structures that colocalize with Dscam::GFP. When expressed along with DscamΔCyto::GFP (middle), Abl::Myc does not redistribute, displaying a similar pattern to when Abl::Myc is expressed alone (bottom). This is quantified using Manders' Correlation Coefficient. M1 presents a measure of the fraction of Abl::Myc that overlaps with Dscam(ΔCyto)::GFP, while M2 presents a measure of the fraction of Dscam(ΔCyto)::GFP that overlaps with Abl::Myc. Both M1 and M2 are significantly increased in Abl-Dscam coexpression when compared to Abl-DscamΔCyto coexpression. Scale bar is 5 μm.

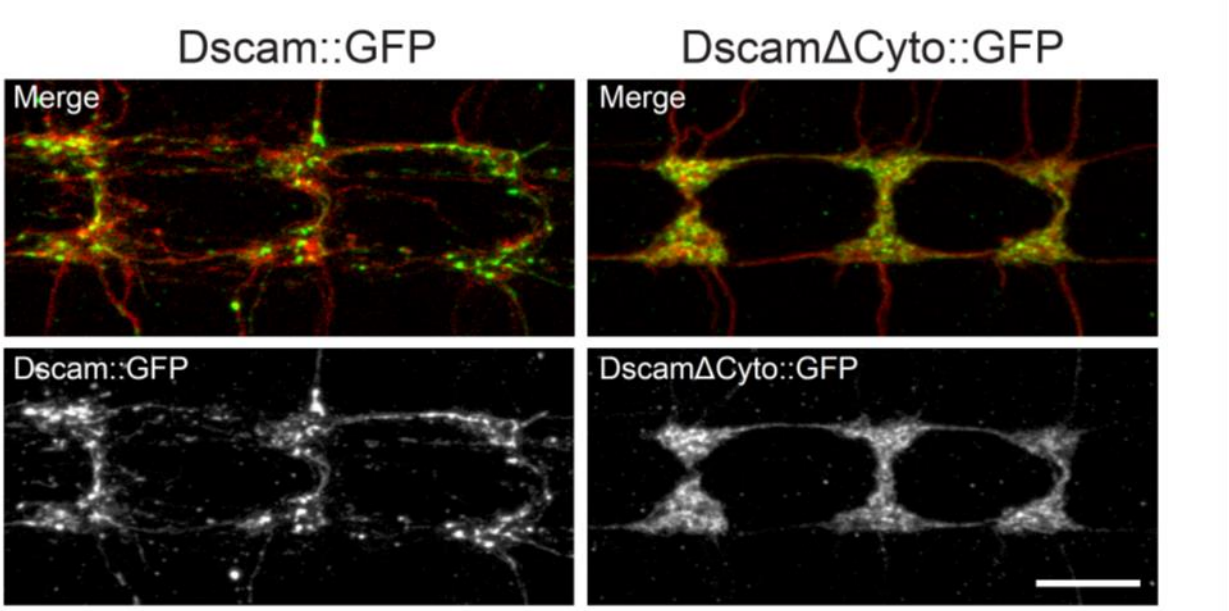

Figure 2.6 DscamΔCyto::GFP is trafficked to presynaptic terminals at a similar level to Dscam::GFP.
Both Dscam::GFP (left) and DscamΔCyto::GFP (right) are trafficked to presynaptic terminals. In addition, presynaptic terminal overgrowth is observed 100% of the time when Dscam::GFP is overexpressed, while presynaptic terminal overgrowth is never observed when DscamΔCyto::GFP is overexpressed. Top image shows merged images mCD8::mRFP (red) and either Dscam::GFP or DscamΔCyto::GFP (green). Bottom images show Dscam::GFP or DscamΔCyto::GFP only. Scale bar is 10 μm.

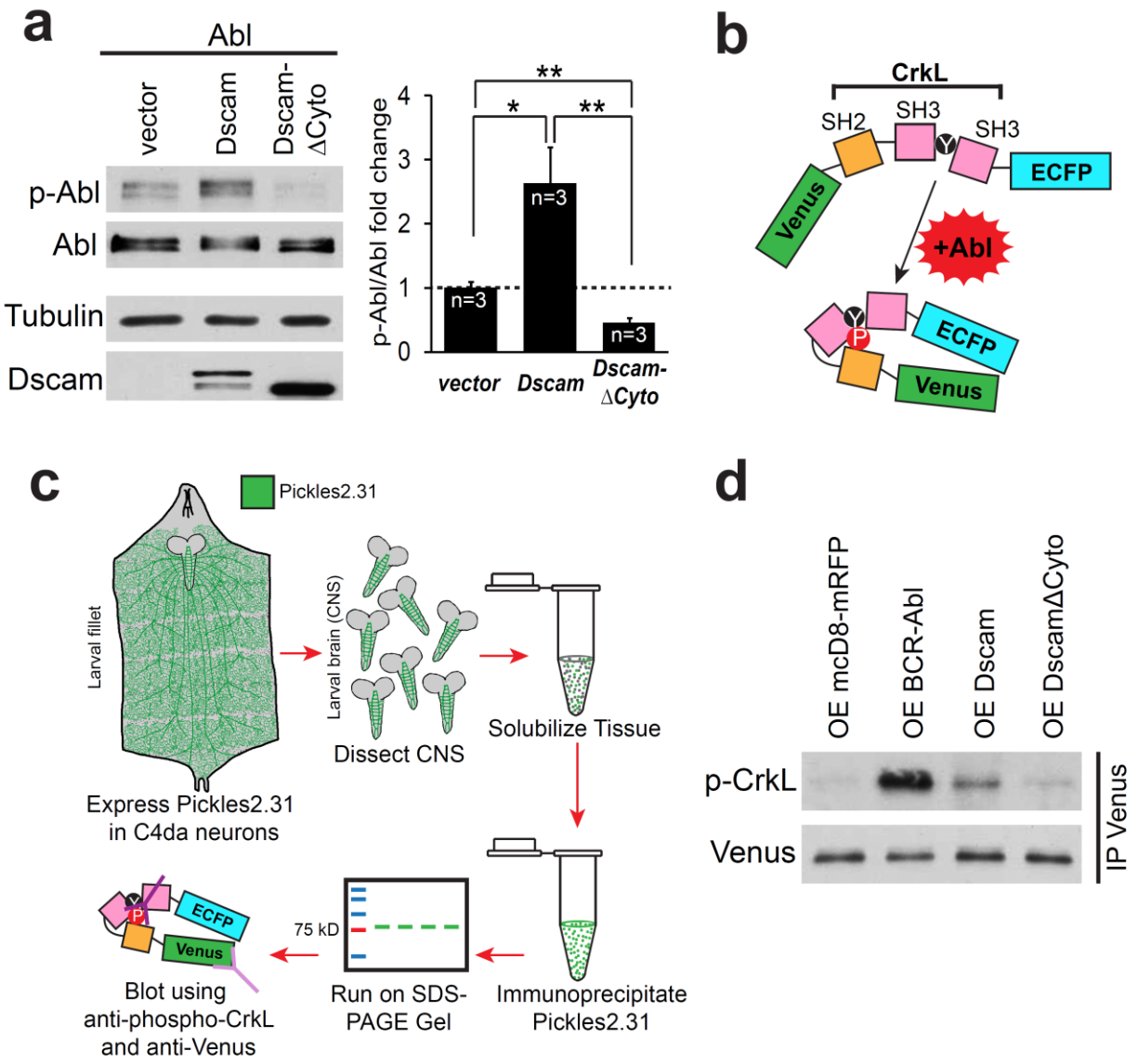

Figure 2.7 Dscam activates Abl kinase in culture and in vivo.
(a) Dscam activates Abl in cultured S2 cells. Abl activation was examined in S2 cell lysates transfected with indicated constructs by using anti-phospho-Y412-Abl antibody. The intensity of phospho-Abl was quantified, normalized to total Abl::Myc, and presented as bar graph (n = 3) (A, right). (b) Schematic of Pickles2.31, an Abl activity reporter that uses phosphorylation of CrkL to report Abl kinase activity. Pickles2.31 is composed of a fragment of human CrkL that contains an Abl phosphorylation site, Y207, sandwiched between ECFP and Venus. Phosphorylation of Pickles2.31 by Abl can be detected with an anti-phospho-Y207-CrkL (p-CrkL) antibody. (c) Schematic of in vivo assay for detecting Abl activity in C4da presynaptic terminals. Pickles2.31 is specifically expressed in C4da neurons. As can be appreciated from the larval fillet diagram (left), the cell bodies and dendrites of C4da neurons reside in the larval body wall while their presynaptic terminals reside in the CNS. To assay Abl activity only in presynaptic terminals, larval CNS are dissected out and solubilized into lysates. Pickles2.31 in the presynaptic terminals is then immunoprecipitated with an anti-Venus antibody (left). After

running on an SDS-PAGE gel, Pickles2.31 expression level can be assayed using an anti-Venus antibody, while the phosphorylation of Y207, a proxy for Abl activity level, can be ascertained by western blotting with a p-CrkL antibody. (d) Dscam activates Abl in presynaptic terminals in vivo. Overexpression of BCR-Abl leads to a robust increase in p-CrkL staining of Pickles2.31 when compared to the mCD8-mRFP control. Similarly, overexpression of Dscam leads to consistent, though less extreme, increase in p-CrkL when compared to control. In contrast, overexpression of DscamΔCyto is indistinguishable from the mCD8-mRFP control. This is a representative blot of three experimental repeats.

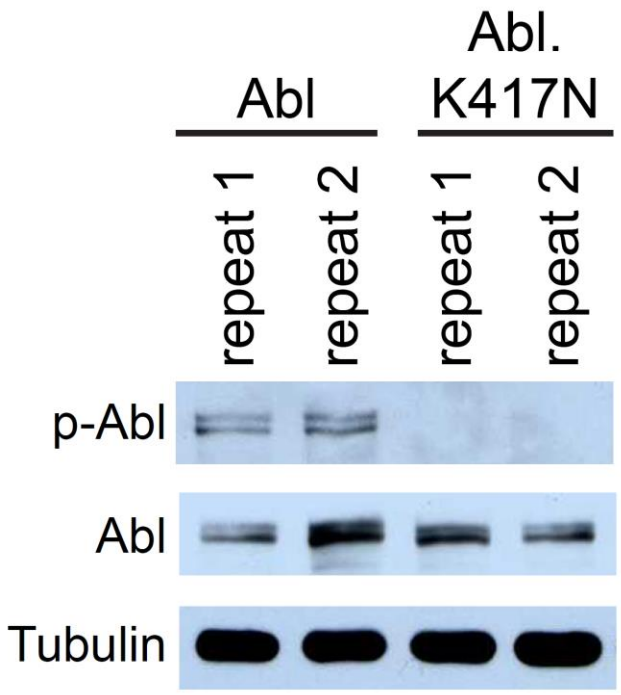

Figure 2.8 Phospho-Y412-Abl antibody specifically reports Abl activation.
S2 cells were transfected with either Abl::Myc or Abl-K417N::Myc. Myc was blotted to report total Abl::Myc or Abl-K417N::Myc level (middle), while phosho-Y412-Abl (p-Abl) was blotted to report Abl kinase activation (top). While Abl::Myc displays a characteristic two-band pattern at the correct molecular weight when blotted for p-Abl, no signal is detected for Abl-K417N. This demonstrates that p-Abl specifically reports Abl activation.

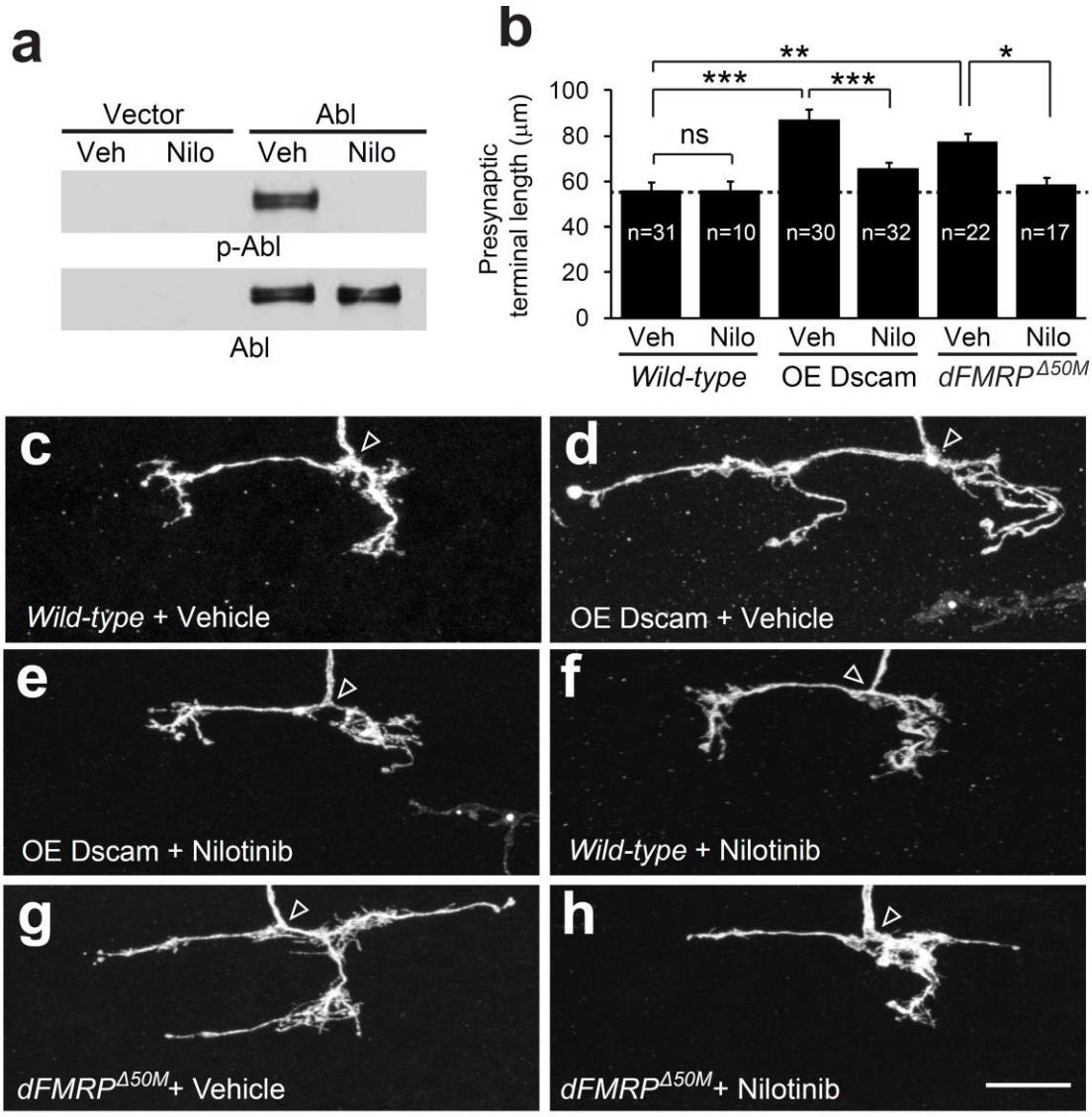

Figure 2.9 Pharmacological inhibition of Abl mitigates the neuronal defects caused by increased Dscam expression in vivo.
(a) Nilotinib inhibits Drosophila Abl kinase. S2 cells were transfected with either Myc-vector or Abl::Myc, and then treated with either vehicle (DMSO) or 5 μM nilotinib for 6 hr. Total lysates were subjected to western blot analysis with phospho-Y412-Abl (p-Abl) (top) and Myc antibodies (bottom). (b) Quantification of the presynaptic terminal length of the indicated genotypes and drug treatment. Sample number is shown inside each bar. (c–h) Nilotinib treatment mitigates presynaptic arbor enlargement caused by Dscam overexpression (OE Dscam, d and e) and by dFMRP mutations (dFMRPΔ50M, g and h). Nilotinib treatment alone does not affect presynaptic terminal growth (f). The arrowhead in each panel points to the location where an axon elaborates the presynaptic terminal arbor. The MARCM technique was used to generate and visualize single presynaptic terminals of mutant C4da neurons. Drosophila larvae were raised in the presence of either 380 μM nilotinib or vehicle (DMSO) for 4 days before the analysis. Scale bar is 10 μm.

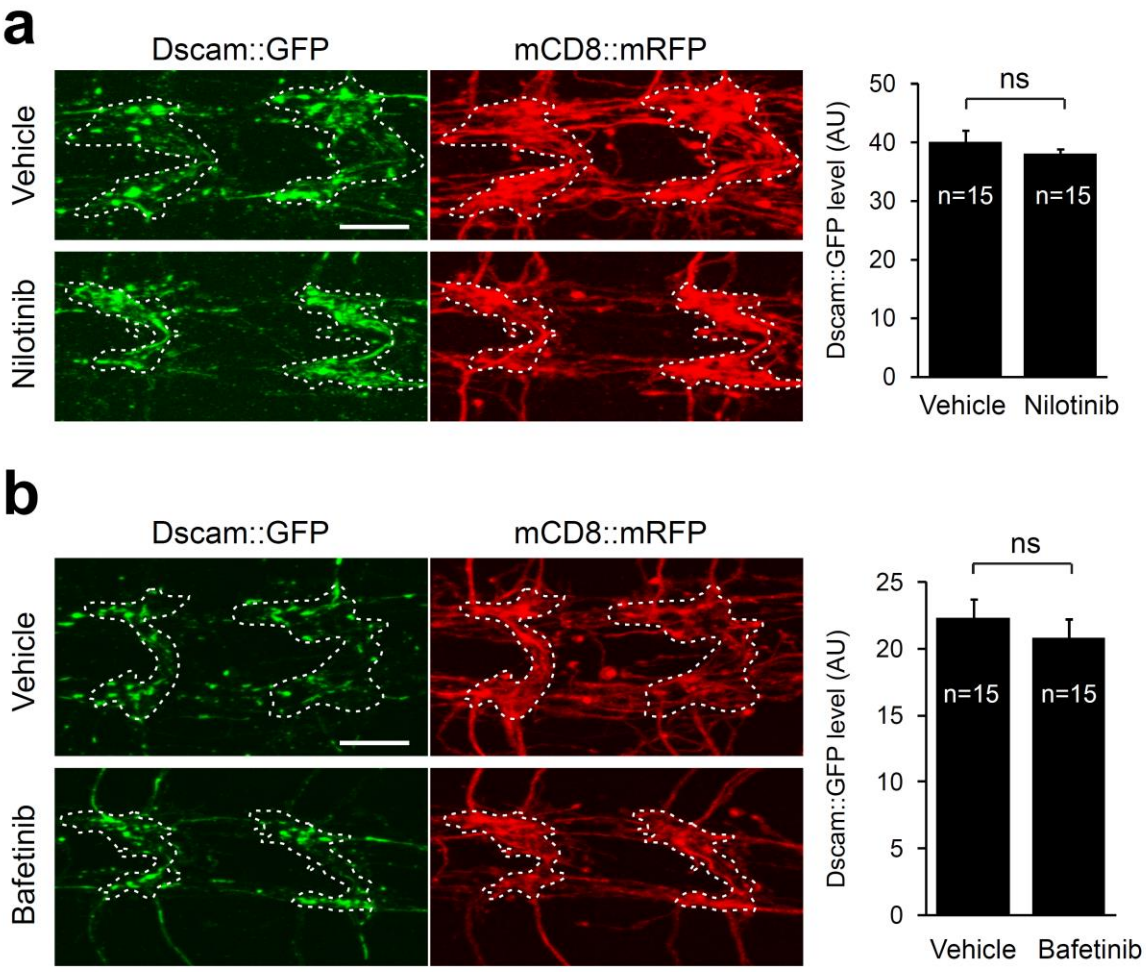

Figure 2.10 Nilotinib and bafatinib do not reduce Dscam transgene expression.
Example images of C4da presynaptic terminals expressing Dscam::GFP in animals fed either vehicle (a and b, top), 380 μM nilotinib (a, bottom), or 125 μM bafetinib (b, bottom) throughout larval development. Images of mCD8::mRFP are shown to indicate the neuropil regions used for the quantifications (white dotted line). Scale bar is 10 μm. Quantification of the fluorescence of the Dscam::GFP transgene in neuropil region is shown on the right. Sample number is shown inside each bar.

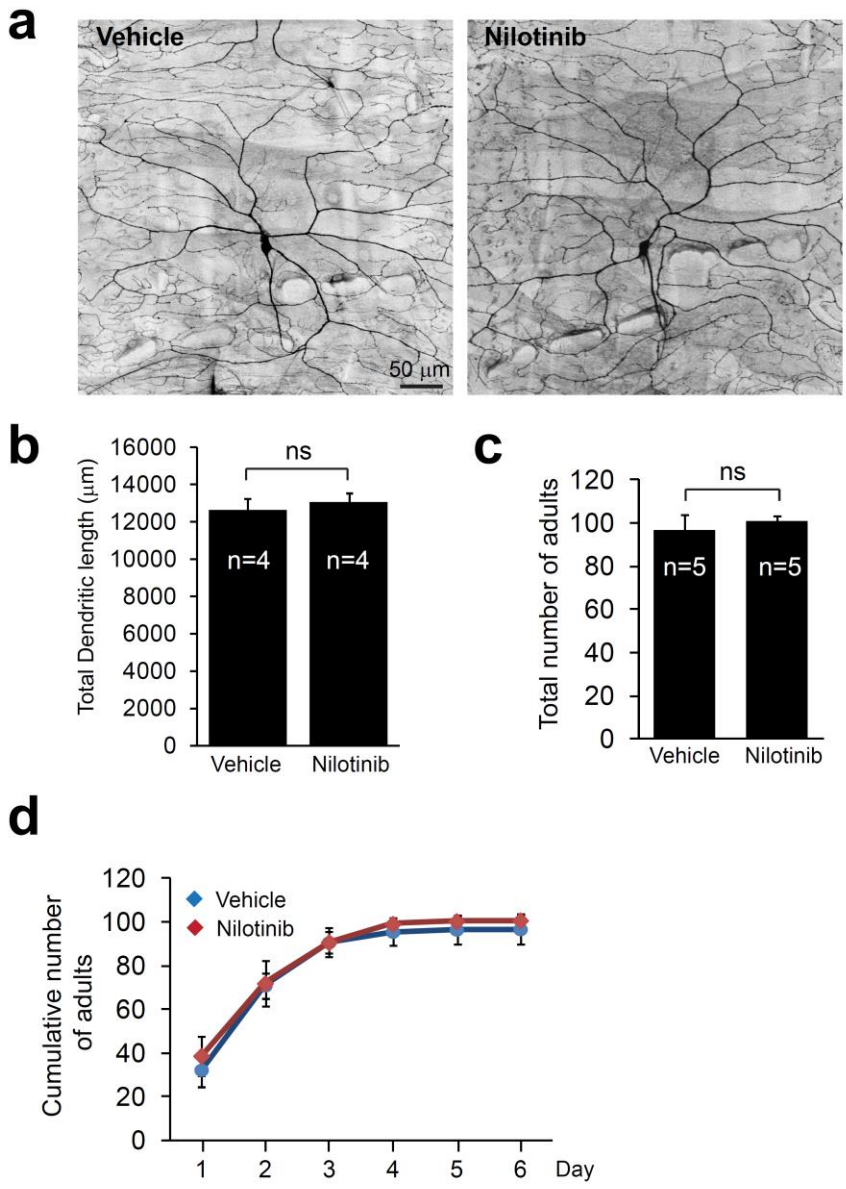

Figure 2.11 Nilotinib treatment does not cause defects in dendritic development or adult viability.

(a and b) Nilotinib does not affect dendritic development. After egg collection, the animals were raised on food containing either vehicle (DMSO) or 380 µM nilotinib for 4 days. C4da dendrites were visualized by expressing mCD8::GFP with ppk-Gal4 (a). Total dendritic length was measured, quantified, and presented in the bar graph (b). Sample number is shown inside each bar. Scale bar is 50 µm. (c and d) Nilotinib does not affect the development of the flies. After egg collection, the animals were raised on food containing either vehicle (DMSO) or 380 µM nilotinib. Eclosed adults were counted on a daily basis. Total number and cumulative number of adults are shown in (c) and (d) respectively.

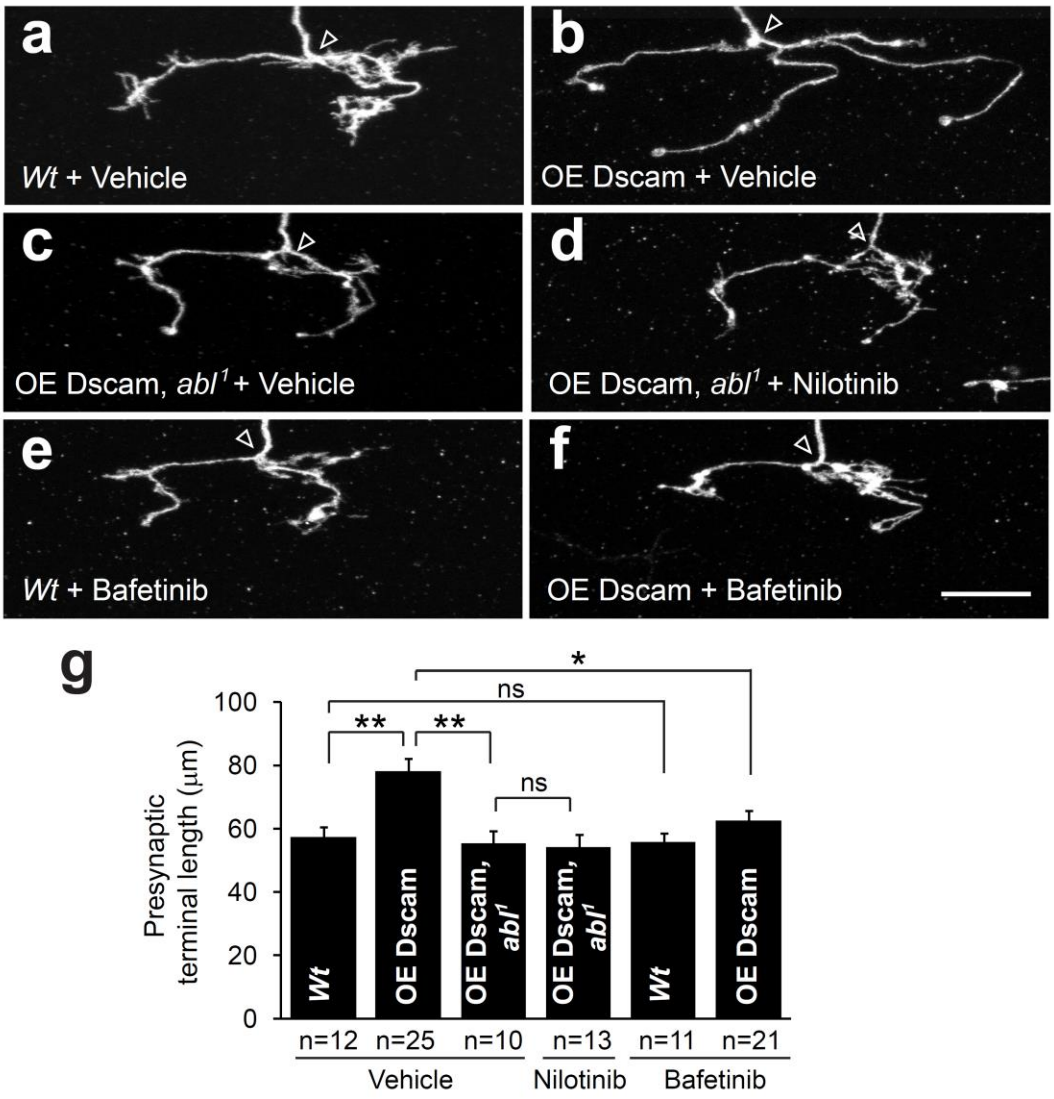

Figure 2.12 Nilotinib and bafetinib act through Abl inhibition to mitigate Dscam-induced presynaptic arbor enlargement in vivo.
The MARCM technique was used to generate and visualize single presynaptic terminals of mutant C4da neurons. Drosophila larvae were raised in the presence of 380 μM nilotinib, 125 μM bafetinib, or vehicle (DMSO) for 4 days before the analysis. Scale bar is 10 μm. (a–d) Nilotinib acts through Abl inhibition to mitigate presynaptic arbor enlargement in Dscam overexpressing neurons. Wt (wild-type, FRT2A), OE Dscam (overexpression of Dscam), OE Dscam, abl1 (overexpression of Dscam in abl1 homozygous mutations). Note that nilotinib does not further decrease the size of presynaptic arbors in abl1 neurons overexpressing Dscam (c and d). (e and f) Bafetinib mitigates presynaptic arbor enlargement in Dscam overexpressing neurons. (g) Quantification of the presynaptic terminal length of the indicated genotype and drug treatment. Sample number is shown below the x-axis.

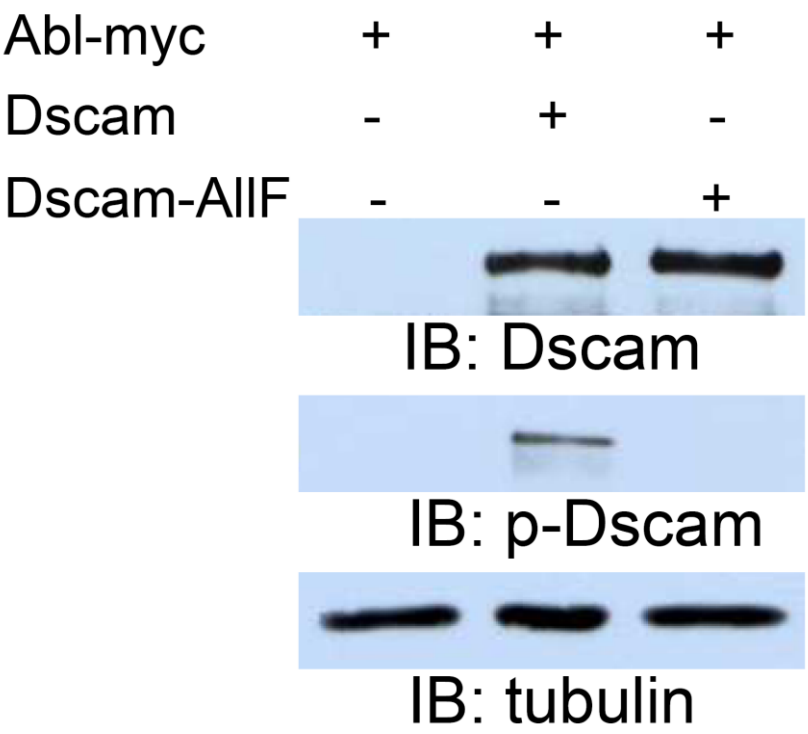

Figure 2.13 Abl phosphorylates a tyrosine residue in the Dscam cytoplasmic domain. While co-expression of Dscam and Abl leads to a band that is recognized by a phospho-specific antibody and matches the size of Dscam, co-expression of Dscam with all cytoplasmic tyrosines mutated to phenylalanine (Dscam-AllF) with Abl does not produce this band. This suggests that Abl phosphorylates Dscam on a cytoplasmic tyrosine.

2.6 Description of manuscript and authors' contributions

The experiments described in Chapter 2 of this dissertation have been published and are available in the form of the following citation:

Sterne, G.R.*, Kim, J.H.*, and Ye, B. (2015). Dysregulated Dscam levels act through Abelson tyrosine kinase to enlarge presynaptic arbors. eLife 4. (* denotes co-first authors)

The relative contributions of each author to the above work is as follows:

GRS, Conceived the project and designed the experiments, Performed the Abl and Dscam overexpression experiments, the mosaic Abl loss-of-function experiments, the dendritic crossing experiments, the colocalization experiments, and the Abl binding and activation biochemistry experiments. Generated figures 2.1-2.8 and 2.13. Wrote the paper

JHK, Conceived the project and designed the experiments, Performed the pilot experiments on Abl overexpression, and performed the pharmacological studies. Generated figures 2.9-2.12. Helped to write the paper.

BY, Conceived the project and designed the experiments, Supervised the project, helped to write the paper.

Chapter 3 Dscam and APP-like cooperate to control presynaptic arbor size

3.1 Abstract

Previous studies suggest that Dscam and APP levels are increased in Down syndrome and may be increased in Fragile X syndrome. Furthermore, increased levels of Dscam or APP promote axon terminal growth in separate systems and require Abl. Despite these similarities, it is not clear whether Dscam and APP interact with one another to influence axon development. We show here that the *Drosophila* homolog of APP, Appl, promotes presynaptic arbor growth in a system that was previously used to study the role of Dscam in presynaptic arbor size control. Furthermore, we show that Dscam and Appl are mutually required and that Appl requires Abl to promote presynaptic arbor growth in this system. Finally, we show that simultaneous increases in Dscam and Appl expression leads to a synergistic presynaptic arbor phenotype, where presynaptic arbor growth is increased beyond that seen when Dscam or Appl are increased individually. These results suggest that Dscam and Appl may interact to control Abl activation and concurrent increases in Dscam and Appl might contribute to the pathogenesis of disorders like Down syndrome and Fragile X syndrome.

3.2 Introduction

3.2.1 Potential interactions of Dscam with other molecules

Several lines of evidence from our studies and others suggest that other molecules may be involved in translating Dscam expression level into presynaptic arbor size. First, I found that

overexpression of DsamΔCyto appears to act as a dominant-negative, decreasing levels of Abl phosphorylation below wildtype levels. However, the mechanism through which Dscam would act as a dominant negative is unclear, suggesting that other molecules may be involved in this pathway. Furthermore, Abl is known to promote axon growth in several other systems and to interact with cell adhesion molecules other than Dscam, suggesting that several separate or intertwined pathways may converge on Abl to control presynaptic arbor size (Leyssen et al., 2005; Soldano et al., 2013). Two scenarios could be imagined: multiple upstream molecules could act separately to control Abl activation, interacting only through their convergent influence on Abl, or Dscam could cooperate with another cell adhesion molecule as coreceptors that together modulate Abl activity. With these possibilities in mind, I noticed that Amyloid Precursor Protein-like (Appl) has previously been shown to require Abl to promote axon and presynaptic arbor growth in two different *Drosophila* systems (Leyssen et al., 2005; Soldano et al., 2013). Appl is the *Drosophila* homolog of Amyloid Precursor Protein (APP), which is known to be an important player in the pathogenesis of Alzheimer's disease. I thus wondered whether Dscam might interact with Appl to control Abl activation and this presynaptic arbor growth.

3.2.2 Down syndrome and Alzheimer's disease

Alzheimer's disease was first described in 1907 by Dr. Alois Alzheimer, who performed an autopsy on a 55-year-old woman who had died from a progressive cognitive and behavioral disorder. Alzheimer found two distinctive pathological features in her brain, neurofibrillary tangles and neuritic plaques. At the time, he correctly inferred that the observed neurofibrillary tangles were abnormal intracellular aggregates and described the neuritic plaques as misshapen neuronal processes surrounding "pathological metabolic substance"(Alzheimer et al., 1995). It was not until later that the observed neurofibrillary tangles were shown to be composed of hyper-

phosphorylated tau and the neuritic plaques of a 4.2 kDa peptide, 40 or 42 amino acids in length (Glenner and Wong, 1984). Glenner and Wong correctly surmised that this 4.2 kDa peptide was likely cleaved from a larger precursor, which was confirmed in 1987 when the amyloid precursor protein (APP) was cloned (Kang et al., 1987). The pathological substance observed by Alzheimer has subsequently become known as the amyloid-β peptide, or Aβ peptide.

In 1968, the link between the amount Aβ deposition and the risk of dementia was established (Blessed et al., 1968), and the disorder that Alzheimer had considered to be a "special illness" is now recognized as a common dementing disorder, currently afflicting 26 million people, with that number expected to quadruple by year 2050 (Brookmeyer et al., 2007). Furthermore, Alzheimer's disease is thought to account for 40-70% of the cognitive variance that is seen in the elderly (Dolan et al., 2010). Among people 71 years old and older in the United States, 16 percent of women have Alzheimer's disease and 11 percent of men have Alzheimer's disease (Plassman et al., 2007; Seshadri et al., 1997).

The prevalence of Alzheimer's disease is much higher in Down syndrome patients, with an earlier age of onset. Down syndrome patients with complete trisomy of chromosome 21 universally develop the amyloid plaques and neurofibrillary tangles by the age of 40. Mean age of Alzheimer's diagnosis in people with Down syndrome is 55, and about 95% of Down syndrome patients develop dementia by the age of 68 (McCarron et al., 2014). This striking phenomenon is thought to arise from the increased copy number of APP, which is located on the duplicated chromosome 21. Consistent with this idea, the alteration in APP gene dosage leads to an approximately 1.5-fold increase in APP mRNA transcript abundance in Down syndrome brains (Oyama et al., 1994). Furthermore, duplication of the APP locus in patients without Down syndrome causes autosomal dominant early-onset Alzheimer's disease, where duplication of a

locus including APP and only 4 other genes was sufficient for the development of Alzheimer's disease pathology (Rovelet-Lecrux et al., 2006). Finally, partial trisomy of chromosome 21 distal to 21q and therefore excluding APP does not lead to the development of Alzheimer's disease pathology, further supporting the idea that three copies of APP drive the development of early-onset Alzheimer's disease in Down syndrome (Prasher et al., 1998).

3.2.3 Physiological functions of APP

APP is part of a small gene family that includes *APLP1* and *APLP2* in humans, *APP-like* (*Appl*) in *Drosophila* and *apl-1* in *C. elegans*. Though *APP* is evolutionarily conserved from worms to humans, the physiological function of APP is still hotly debated. In particular, the initial phenotype of *APP*-deficit mice was rather underwhelming. These mice were viable and fertile though they had lighter body mass, age-related weakness in the extremities, and sporadic reactive gliosis was observed in the brain (Zheng et al., 1995). Further analysis has since revealed subtle defects in exploratory behavior and spatial learning (Tremml et al., 1998). However, double mutant of members of the APP gene family produced more compelling phenotypes, suggesting that the members of the APP gene family are essential genes with overlapping functions. Alone, APLP1-deficient mice have a postnatal growth defect (Heber et al., 2000) and APLP2-deficient mice appear completely normal (von Koch et al., 1997). In contrast, APP/APLP2 or APLP1/APLP2 double knockouts exhibited early postnatal lethality (Heber et al., 2000; von Koch et al., 1997). The final dual gene deficiency, APP/APLP1, was viable, fertile and without additional abnormalities as compared the single knockouts (Heber et al., 2000). Taken together, these results suggest that the APP gene family is essential for normal development and survival.

What is the normal physiological function of APP gene family members? One of the earliest APP functions to be discovered was its role in cellular growth, where decreased expression of APP slows fibroblast growth (Saitoh et al., 1989). This function was later mapped to the pentapeptide (RERMS) domain of APP, which is located near the middle of the extracellular domain (Ninomiya et al., 1993). Infusion of either the pentapeptide or secreted APP into the brain increased synaptic density and improved memory retention in mice and rats (Meziane et al., 1998; Roch et al., 1994). A role for APP in synaptic development is supported by the finding that APP/APLP2 double knockout mice have defects in neuromuscular junction (NMJ) formation and show aberrant apposition of pre- and post-synaptic proteins. The number of synaptic vesicles at each active site was also found to be reduced (Wang et al., 2005). The importance of APP for synapse formation is also conserved in *Drosophila*, where loss of Appl causes a decrease in bouton number in the NMJ. Congruently, overexpression of Appl in the *Drosophila* NMJ leads to increased bouton number and alterations in synaptic structure (Torroja et al., 1999b).

In addition to promoting synapse formation, APP stimulates neurite outgrowth in a variety of settings (Hoe et al., 2009; Koo et al., 1993; Qiu et al., 1995; Small et al., 1994; Young-Pearse et al., 2008). Consistent with a role of APP in normal neuronal development, upregulation of APP expression is seen during neuronal maturation of primary hippocampal cultures (Hung et al., 1992). This upregulation during development is also seen in studies in *Drosophila*, where Appl is enriched areas of synapse formation and in growing axons (Torroja et al., 1996). Relatedly, Appl has been shown to play a role in axonal outgrowth in *Drosophila*, where increased expression of human APP and *Drosophila* Appl lead to increased arborization of small lateral ventral neurons in the adult central nervous system (Leyssen et al., 2005). This

conservation of function from mammals to invertebrates also argues for conserved function of APP family members in development. Furthermore, previous studies suggest that, like Dscam, Appl signals through Abl. In the adult *Drosophila* small lateral ventral clock neurons (sLNv), Abl is required for Appl to promote axonal arborization. In this system, overexpression of human APP leads to increased axonal arborization, which can be mitigated by either the removal of a single copy of *abl* or by simultaneous overexpression of a kinase-dead form of Abl (Leyssen et al., 2005). In another adult *Drosophila* cell type, the mushroom bodies, Appl is required for both α- and β-axon growth. Simultaneous loss of both copies of *Appl* and one copy of *abl* led to a more penetrant loss of β-axons, and overexpression of Abl in mushroom bodies lacking Appl rescued β-axon growth, suggesting that Abl is downstream of Appl in mushroom body axon growth (Soldano et al., 2013). Given that both Dscam and Appl promote neurite growth, it is especially intriguing that both of these genes are upregulated in both Down syndrome and Fragile X syndrome.

3.2.4 APP expression is increased in Down syndrome and Fragile X syndrome

Analysis of partial trisomy 21 patients suggests that APP is not required for the development of intellectual disability in Down syndrome but suggested that its duplication may contribute to the intellectual disability phenotype (Korbel et al., 2009). As mentioned earlier, increased APP mRNA is found in the brains of Down syndrome patients, where it is suspected to contribute to the development of early-onset Alzheimer's disease (Oyama et al., 1994). In Fragile X syndrome, studies in mice show that FMRP binds to and regulates the translation of APP (Westmark and Malter, 2007). Importantly, loss of FMRP leads to increased APP expression, and levels of soluble $A\beta_{40}$ and $A\beta_{42}$ are increased in *FMR1* knockout mice (Westmark and Malter, 2007). Interestingly, levels of secreted APP are two or more times higher in children with

severe autism than in those without autism, and the amount of secreted APP correlated with symptom severity, such that children with severe autism had higher levels of secreted APP than children with mild autism (Sokol et al., 2006). Taken together, these data suggest that APP is likely to be increased in both Down syndrome and Fragile X syndrome, where it may contribute to development of intellectual disability in each disorder.

In this chapter I show that Appl promotes presynaptic arbor growth in C4da presynaptic arbors. Furthermore I show that Dscam and Appl are mutually required to promote presynaptic arbor growth and that Appl also requires Abl in C4da neurons. In addition, I present evidence that the mechanism through which Appl influences presynaptic arbor growth may not be through protein trafficking. In addition, I show that Appl requires the conserved E1 extracellular domain to promote presynaptic arbor growth. Finally, I show that Dscam and Appl act synergistically to promote presynaptic arbor growth when expression of both proteins is increased, suggesting that simultaneously increased Dscam and Appl levels may lead to more severe consequences in Down syndrome and Fragile X syndrome than changes in either of these proteins alone.

3.3 Materials and Methods

3.3.1 Fly strains

abl^{1} (Gertler et al., 1989), *abl*^{4} (Bennett and Hoffmann, 1992), *Appl*^{d} (Luo et al., 1992), *Dscam*^{18} (Wang et al., 2002), *Dscam*^{P1} (Schmucker et al., 2000), ppk-Gal4 (Kuo et al., 2005), UAS-Dscam[3.36.25.2]::GFP (Yu et al., 2009), UAS-Appl, UAS-Appl.sd, UAS-Appl.s (Luo et al., 1992; Torroja et al., 1996), UAS-Appl.sd, UAS-Appl.sd.ΔNPTY, UAS-Appl.sd.ΔCg, UAS-Appl.sd.ΔE1, Appl.sd.ΔE2 (Torroja et al., 1999b), UAS-APP695-N-myc, and UAS-APP695.SPA4.CT.T-N-myc (Fossgreen et al., 1998) were used in this study.

3.3.2 Labeling presynaptic terminals using MARCM

The MARCM technique was used to visualize single neurons homozygous for *abl*^{1}, *abl*^{4}, *Dscam*^{18}, *Dscam*^{P1}, or *Appl*^{d}, and overexpressing Dscam[3.36.25.2]::GFP or Appl as previously described (Kim et al., 2013).

3.3.3 Immunostaining and imaging

Immunostaining of third-instar larvae was accomplished as previously described (Ye et al., 2011). Antibodies used include chicken anti-GFP (Aves, Tigard, Oregon) and rabbit anti-RFP (Rockland, Limerick, Pennsylvania). Samples were dehydrated and mounted with DPX mounting media (Electron Microscopy Sciences, Hatfield, Pennsylvania). Confocal imaging was completed with a Leica SP5 confocal system equipped with a resonant scanner and 63× oil-immersion lens (NA = 1.40). Images were collected and quantified as previously described (Kim et al., 2013).

3.3.4 Statistical analysis

Two-way student's t- test was used for statistical analysis. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$; ns: not significant.

3.4 Results

3.4.1 Appl promotes presynaptic arbor growth in C4da neurons

In order to ask whether overexpression of Appl promotes presynaptic arbor growth in C4da neurons, I overexpressed Appl under the control of the UAS promoter in all C4da neurons using ppk-Gal4. Consistent with previous reports, increased expression of Appl led to a robust increase in presynaptic arbor growth (Figure 3.5 b).

3.4.2 Dscam and Appl are mutually required to promote presynaptic arbor growth

Since overexpression of Appl and overexpression of Dscam produce similar phenotypes, I next asked whether Dscam requires Appl to instruct presynaptic arbor growth. Consistent with previous experiments, overexpression of Dscam in single C4da neurons using MARCM led to a significant increase in presynaptic arbor length (Figure 3.1 b,e). Furthermore, $Appl^{d}$ mutant clones alone were significantly shorter than wildtype (Figure 3.1 c,e), suggesting that Appl instructs presynaptic arbor size in C4da neurons. $Appl^{d}$ mutant clones that overexpressed Dscam, however, did not differ significantly in length from $Appl^{d}$ clones, and were significantly shorter than clones that overexpressed Appl in a wildtype background (Figure 3.1 d,e). These results suggest that Appl instructs presynaptic arbor growth in C4da neurons and that Dscam requires Appl to instruct presynaptic arbor growth.

Although these results indicate that Dscam requires Appl, they do not distinguish between two possible scenarios: that Appl is downstream of Dscam, and that Dscam and Appl are mutually required. To assess the relationship of Appl to Dscam in presynaptic arbor size control, I next asked whether Appl requires Dscam to instruct presynaptic arbor growth. Overexpression of Appl in single clones led to an extremely robust increase in presynaptic arbor length (224%) as compared to wildtype clones (Figure 3.2 a,b,g). Consistent with previous reports, $Dscam^{18}$ mutant clones had severely truncated presynaptic arbors, and were highly significantly shorter than wildtype clones (Figure 3.2 c,g). $Dscam^{18}$ mutant clones that overexpressed Appl were significantly shorter than wildtype clones overexpressing Appl, but remained slightly but significantly longer than $Dscam^{18}$ mutant clones that did not overexpress Appl (Figure 3.2 d,g). To confirm this result, I repeated these experiments using a different mutant allele of *Dscam*, $Dscam^{P1}$. Consistent with the results obtained using $Dscam^{18}$, $Dscam^{P1}$

clones that overexpressed Appl were significantly shorter than wildtype clones that overexpressed Appl, but remained slightly but significantly longer than *Dscam*^{P1} clones that did not overexpress Appl (Figure 3.2 e,f,g). These results suggest that Appl also requires Dscam to instruct presynaptic arbor growth, and therefore Appl and Dscam are mutually required to control presynaptic arbor size. Furthermore, these results suggest that Appl also exerts a portion of its effects on presynaptic arbor size through another, yet unidentified, pathway that is independent of Dscam.

3.4.3 Appl requires Abl to promote presynaptic terminal growth

Next, since Appl requires Dscam to instruct presynaptic arbor size, I asked whether Abl is also required by Appl in C4da neurons. To do this, I overexpressed Appl in either wildtype or *abl*^{1} single C4da neurons using MARCM. Compared to single C4da neurons overexpressing Appl, which had significantly and extremely robustly increased presynaptic arbor lengths as compared to wildtype (715%) (Figure 3.3 b, g), *abl*^{1} mutant clones that overexpressed Appl were significantly shorter (Figure 3.3 d,g). Importantly, *abl*^{1} mutant clones that overexpressed Appl were significantly longer than *abl*^{1} clones that did not overexpress Appl (Figure 3.3 c,d,g). This result was confirmed using an independent mutant allele of *abl*, *abl*^{4}. Taken together, these results suggest that Appl requires Abl to instruct presynaptic arbor size, but that Appl also signals through another, yet unidentified, pathway to affect presynaptic arbor size.

3.4.4 Constitutively secreted Appl is not sufficient to promote presynaptic arbor growth

I next asked which portions of the Appl molecule are required to promote presynaptic terminal growth. To do this, I overexpressed a previously generated collection of mutated UAS-Appl constructs in C4da neurons using ppk-Gal4 (Torroja et al., 1999b). Appl is known to be proteolytically processed by cleavage at identified sites in the extracellular domain, which leads

to the release of soluble forms of Appl (Fossgreen et al., 1998; Luo et al., 1992; Torroja et al., 1996). I thus asked whether a constitutively secreted form of Appl, Appl.s or secretion deficient form of Appl, Appl.sd could promote presynaptic terminal growth. Appl.s is a form of Appl that lacks the cytoplasmic and transmembrane domains and is thus constitutively secreted (Figure 3.4). Overexpression of Appl.s did not lead to an appreciable change in presynaptic arbor size, and if anything may decrease the number of connectives, though further analysis with MARCM would be necessary to confirm this phenotype (Figure 3.5 c). Appl.sd is a secretion-deficient form of Appl that has a 34 amino-acid in-frame deletion (amino acids 758-791) as well as two point mutations ($Arg_{795}Arg_{796}$ – $Leu_{795}Ser_{796}$). The point mutations alone are not sufficient to prevent Appl secretion, suggesting that the 34 amino acid deletion contains the sites necessary for Appl processing. In contrast to Appl.s, Appl.sd was able to promote presynaptic arbor growth, though not to the same extent as wildtype Appl (Figure 3.5 d). These data suggests that secreted Appl does not promote presynaptic arbor growth, but that the region deleted in Appl.sd or the associated point mutations may be necessary for one, but not all, of the signaling pathways through which Appl impacts presynaptic arbor growth.

3.4.5 Appl requires the E1 extracellular domain to promote presynaptic arbor growth

I next tested which domains of Appl were required to promote presynaptic arbor growth using a series of mutants, all of which had the *.sd* mutation to examine the secretion-independent roles of Appl. I focused on conserved domains that were identified by comparing APP and Appl (Rosen et al., 1989). These include a conserved internalization sequence in the cytoplasmic domain (GYENPTY) (Appl.sdΔNPTY) and two extracellular domains with high sequence homology with APP, E1 (Appl.sdΔE1) and E2 (Appl.sdΔE2). I further tested the conserved G_O binding site (Appl.sdΔCg), which has been demonstrated to play a role in the signaling of both

APP and Appl (Nishimoto et al., 1993; Okamoto et al., 1995). The constructs have the following deletions: Appl.sdΔNPTY which lacks the GYENPTY internalization sequence and surrounding amino acids (872-883), Appl.sdΔE1 deletes the distal half of the E1 as well as a number of amino acids located on the c-terminal side of E1 (85-321 aa), Appl.sdΔE2 deletes 75% of the distal region of E2 as well as some amino acids c-terminal to it (449-740 aa), and Appl.sdΔCg, which deletes the putative G_O-protein binding site (845-855 aa) (Figure 3.4). These constructs have previously been demonstrated to be expressed and at comparable levels to one another (Torroja et al., 1999b). I overexpressed these constructs in all C4da neurons under the control of ppk-Gal4.

Overexpression of Appl.sdΔNPTY did not lead to a decrease in presynaptic terminal growth as compared to Appl.sd but did lead to an additional neuropil phenotype, suggesting that the internalization sequence may be important for proper synapse formation in the connectives (Figure 3.5 e). Overexpression of Appl.sdΔE1, however, failed to promote presynaptic arbor growth (Figure 3.5 g), suggesting that part of the N-terminal half of the extracellular domain is required for Appl to promote presynaptic arbor growth through both Dscam/Abl and through its other signaling pathway(s). In contrast to Appl.sdΔE1, Appl.sdΔE2 did not compromise Appl's action on presynaptic arbor growth and, surprisingly, overexpression of Appl.sdΔE2 was indistinguishable from that of wildtype Appl (Figure 3.5 h). This result is unexpected because deletion of the E2 domain appears to rescue the defects caused by the .sd mutation. Finally, overexpression of Appl.sdΔCg resulted in a phenotype that was indistinguishable from overexpression of Appl.sd (Figure 3.5 f). This result suggests that either the G_O-protein binding site is not required for Appl to promote presynaptic arbor growth or that the G_O-protein binding site is involved in the same pathway that is impacted by the .sd deletion. Taken together these

results suggest that the E1 region in particular is important for Appl to promote presynaptic arbor growth and provides further evidence that Appl signals through two or more distinct pathways to regulate presynaptic arbor size, one of which requires Dscam/Abl.

3.4.6 Appl is not required for Dscam/Abl colocalization in C4da presynaptic arbors

Since both Appl and Dscam require Abl, and loss of Appl prevents Dscam from promoting presynaptic arbor growth, we next asked whether Dscam requires Appl to associate with Abl. Our previous work has shown that Dscam::GFP and Abl::myc colocalize in presynaptic arbors in vivo and that this colocalization is dependent on the Dscam cytoplasmic domain. We thus used the same assay to ask whether loss of Appl would decrease colocalization of Dscam::GFP and Abl::myc in presynaptic arbors. To do this, we overexpressed Dscam::GFP and Abl::myc either in a wildtype or an $Appl^{d}$ mutant background and assayed colocalization. As in the wildtype background, co-expressed Dscam::GFP and Abl::myc exhibited nearly complete colocalization in the $Appl^{d}$ mutant (Figure 3.6, middle and bottom). Strikingly, however, we noticed a conspicuous change in Dscam::GFP localization within presynaptic arbors, such that Dscam::GFP became clumped into large puncta (Figure 3.6, bottom). As might be expected, Abl::myc was redistributed to match the location of Dscam::GFP puncta. These results suggest that Appl is not required for Dscam-Abl binding, but that Appl may be important for Dscam localization in presynaptic arbors.

3.4.7 Loss of Appl does not block presynaptic arbor growth by decreasing Dscam trafficking to the presynaptic arbors

Previous studies have suggested that Appl is involved in axonal transport and that Appl functions as a vesicular receptor for kinesin-1 (Gunawardena and Goldstein, 2001; Torroja et al., 1999a). Importantly, these studies showed that loss of Appl led to axon blockages or “organelle

jams" which led to punctate aggregations of the observed proteins, including synaptotagmin and cysteine string protein. Thus, to test whether Appl is required to transport Dscam to the presynaptic arbor, we first asked whether loss of *Appl* altered Dscam expression level and distribution in matched samples that were stained and imaged simultaneously to allow for direct comparison of Dscam expression level. I hypothesized that if Appl is required to transport Dscam to presynaptic arbors that Dscam-GFP should form punctate axon blockages in axon shafts and decreased Dscam-GFP should be observed in the presynaptic arbors in *Appl* mutants. While Dscam-GFP was present in large bright puncta in the axon shafts and presynaptic terminals of *Appl* mutants, Dscam-GFP intensity was far brighter in the presynaptic arbors as compared to controls (Figure 3.7, right). This result suggests that loss of Appl does not impair Dscam trafficking to presynaptic arbors, but rather may enhance it. In addition, it suggests that the reduction of presynaptic arbor growth in *Appl* mutants overexpressing Dscam is unlikely to stem from reduced transport of Dscam to presynaptic arbors.

Previous studies of Appl's role in trafficking also demonstrated that both overexpression of and loss of Appl led to the same axon blockage phenotype, since overexpression of Appl binds up the pool of kinesin-1, preventing kinesin-1 from interacting with other trafficking pathways for which it is required. Importantly, these studies predict that Appl loss-of-function and Appl gain-of-function should result in the same phenotype in systems that rely on Appl's role as a vesicular receptor for kinesin-1 (Gunawardena and Goldstein, 2001). Our system does not seem to fit this model, as gain and loss of Appl function lead to changes in opposite directions. In particular, loss of Appl leads to significantly decreased presynaptic arbor length in single C4da neurons while overexpression of Appl in single C4da neurons leads to extremely significantly increased presynaptic arbor length (Figures 3.1 c,e, Figure 3.2 b,g and Figure 3.3

b,g). Taken together, these data suggests that Appl may not be required for Dscam trafficking in our system and may instead act through a yet undiscovered mechanism.

Finally, previous studies found that while overexpression of wildtype Appl produced axon jams of synaptotagmin and cysteine string protein, overexpression of Appl lacking the cytoplasmic domain, which contains a conserved region that contains a kinesin-1 binding site, failed to produce these jams (Gunawardena and Goldstein, 2001). Furthermore, Appl genetically interacts with kinesin-1 to facilitate axon transport. I reasoned that if Appl's interaction with kinesin-1 is required in our system, Appl lacking its cytoplasmic domain should be unable to promote presynaptic arbor growth. Overexpression of an Appl construct that lacks the kinesin-1 binding domain should be unable to promote presynaptic arbor growth. Although the collection of mutant Appl constructs we tested did not include a mutant which covered the kinesin-1 binding domain, we were able to access a human APP construct lacking the entire cytoplasmic domain. To assess whether the human APP cytoplasmic domain is required for APP to promote presynaptic arbor growth, we first asked whether wildtype human APP could promote presynaptic arbor growth. Consistent with previous reports suggesting that Appl and APP are highly functionally homologous (Luo et al., 1992), overexpression of APP695 also led to increased presynaptic arbor growth (Figure 3.8, b). This result suggests that the functional domains required for Appl's effect on presynaptic arbor growth are conserved in human APP. We next asked whether loss of the cytoplasmic domain, which includes the kinesin-1 binding domain, abolished the increase in presynaptic arbor growth seen when APP was overexpressed (Figure 3.8, a). In opposition to the hypothesis that APP exerts its function through influencing Dscam trafficking, loss of the kinesin-1 binding domain did not impact Appl's ability to promote presynaptic arbor growth. In fact, the phenotype seen in C4da neurons overexpressing APPΔCT

was indistinguishable from that in C4da neurons overexpressing wildtype APP (Figure 3.8, b). Both APP and APPΔCT were expressed in presynaptic arbors as seen by the localization of myc (Figure 3.8, b, right). This result further suggests that Appl may not promote presynaptic arbor growth by increasing Dscam trafficking to the presynaptic arbors.

3.4.8 Simultaneously increased Dscam and Appl have a synergistic effect on presynaptic arbor growth

Finally, since APP and Dscam are predicted to have simultaneously increased expression in Down syndrome and Fragile X syndrome, I asked how simultaneous overexpression of Appl and Dscam would impact presynaptic arbor growth. To do this, I overexpressed Appl and Dscam::GFP together in C4da neurons. I found that overexpression of Appl and Dscam led to an arrestingly synergistic phenotype, which promoted presynaptic arbor growth so strongly that axon arbors left the confined of the C4da axon ladder and grew in all directions (Figure 3.9). These Appl and Dscam overexpressing C4da axon ladders were easily distinguishable from those overexpressing either Appl or Dscam alone (Figure 3.9). This result is significant because it suggests that concurrent increases in APP and Dscam could lead to even more significant changes in axon development than increased levels of either APP or Dscam alone.

3.5 Discussion

In this study, we showed that Dscam and Appl are mutually required to promote presynaptic arbor growth and that Appl, like Dscam, requires Abl. We further showed that simultaneous overexpression of Appl and Dscam has a synergistic phenotype, suggesting that coincident increases of APP and Dscam, as is seen in Down syndrome and Fragile X syndrome, may lead more severe changes in development than increased Dscam or APP alone.

One interesting prediction gleaned from our Dscam-Appl epistasis experiments is that Appl requires Dscam and Abl but may also act through another, yet unidentified signaling pathway. This prediction is supported by our analysis of the Appl.sd mutant construct, overexpression of which led to an intermediate phenotype. This suggests that the region deleted in .sd is required for one pathway, but not all, through which Appl promotes presynaptic arbor growth. We hypothesize that, in addition to Abl, Appl may also signal through the G_O-protein, which has previously been implicated in promoting synaptic bouton formation in the *Drosophila* NMJ (Torroja et al., 1999b). The observation that deletion of the G_O-binding site in the Appl.sd construct does not lead to further reductions in presynaptic arbor growth does not rule out the participation of G_O. If the mutation in .sd is also required for G_O function in our system, further deletion of the G_O-binding site would not lead to further changes in Appl's ability to promote presynaptic arbor growth. However, further experiments are required to determine whether Appl also signals through G_O-protein in C4da presynaptic arbor size control.

Another intriguing question raised by this study relates to the somewhat contradictory phenotypes seen when overexpressing Appl versus APP deletion constructs. Overexpression of the Appl.sd deletion constructs shows that deletion of the conserved GYENPTY internalization (Appl.sdΔNPTY) sequence on the Appl cytoplasmic domain leads to a neuropil phenotype in C4da neurons. In contrast, overexpression of APPΔCT, which also lacks the internalization sequence, was indistinguishable overexpression of APP in C4da neurons. This discrepancy suggests that although APP and Appl seem to share a domain that is capable of promoting presynaptic arborization, not all functions or regions are conserved between APP and Appl. Thus, future studies that utilize APP and not Appl might produce results that are more readily translatable into mammalian systems.

An open question that is not fully addressed by these experiments is the mechanism of the interaction between Appl, Dscam, and Abl. Since loss of Appl increases Dscam levels in presynaptic arbors and overexpression of an APP construct that lacks the kinesin-1 binding domain fully retains its ability to promote presynaptic arbor growth, suggesting that kinesin-1 mediated transport is not required, it is unlikely that Appl is acting to transport Dscam to presynaptic arbors. However, the bright large puncta of Dscam-GFP that are observed in *Appl* mutants may suggest that Appl is important for Dscam turnover or degradation in presynaptic arbors. This possibility remains to be tested but is not mutually exclusive of other hypotheses.

If Appl does not exert its functions by altering Dscam trafficking to presynaptic arbors, an alternative hypothesis is that Appl and Dscam act as co-receptors. Experiments presented here suggest that Appl may not facilitate Dscam-Abl binding, as loss of Appl does not disrupt in vivo colocalization of Dscam and Abl. However, the corresponding hypothesis, that Dscam mediates Appl's interaction with Abl, remains untested. To test this hypothesis, Appl and Abl co-immunoprecipitation could be assessed with and without expression of Dscam. If Dscam is required to link Appl to Abl, expression of Dscam should increase the amount of Abl that co-immunoprecipitates with Appl. Furthermore, if Dscam and Appl act as co-receptors, co-expression of Dscam and Appl should lead to increased activation of Abl when compared to either Dscam or Appl alone. This experiment could be performed by expressing Abl-myc with either Dscam alone, Appl alone, or Dscam and Appl together in *Drosophila* S2 cells and assessing Abl activation using the phospho-Abl antibody employed in our Dscam-Abl study. If Dscam and Appl act as co-receptors, co-expression should lead to increased Abl activation over either Dscam or Appl alone. This experiment may be complicated by the endogenous expression

of Dscam or Appl in S2 cells, in which case RNAi could be employed to knock down either Dscam or Appl expression.

Interestingly, the only domain of Appl that appears to be absolutely required to promote presynaptic growth is the E1 extracellular domain. This domain has previously been shown to be important for activation of G_O-protein (Okamoto et al., 1995) and for APP dimerization (Dahms et al., 2010). However, the deletion construct that I tested covered more than just the E1 domain, raising the possibility that the region found to be required for Appl to promote presynaptic terminal growth has additional functions. In particular, if the experiments described above indicate that Dscam and Appl are co-receptors, it might be hypothesized that Appl binds to Dscam through the large region deleted in Appl.sdΔE1.

Finally, the results presented here suggest that simultaneous overexpression of APP and Dscam, as is seen in Down syndrome and likely also in Fragile X syndrome, leads to a synergistic phenotype. Thus, the increased expression of two components of the same pathway may lead to more severe defects than two components that perform different cellular functions. Furthermore, this study provides a possible explanation for two observations in human patients. These include that duplication of the APP locus leads to the development of early-onset Alzheimer's disease but not intellectual disability (Rovelet-Lecrux et al., 2006), and the identification of APP as a possible contributing factor that was not absolutely required for the intellectual disability phenotype in Down syndrome (Korbel et al., 2009). Korbel et al (2009) identified several partial chromosome 21 trisomy patients with borderline normal intellectual functioning that had three total copies of APP, suggesting that APP may contribute but that three copies of APP is not sufficient for the development of intellectual disability. However, they also noted two patients with four total copies of APP that had more pronounced intellectual disability,

indicating that the contribution of APP may scale with dosage. Thus, the presence of an extra copy of APP in an otherwise normal individual may lead to subtle defects that do not significantly impact intelligence. However, increased copy number of both Dscam and APP may have a synergistic phenotype that noticeably impacts IQ. Finally, the partial dependence of Appl on Abl suggests that a similar therapeutic approach could be used to mitigate the consequences of increased APP expression and increased Dscam expression for presynaptic arbor size. However, whether administration of tyrosine kinase inhibitors rescues presynaptic arbor growth in Appl-overexpressing larvae has yet to be tested.

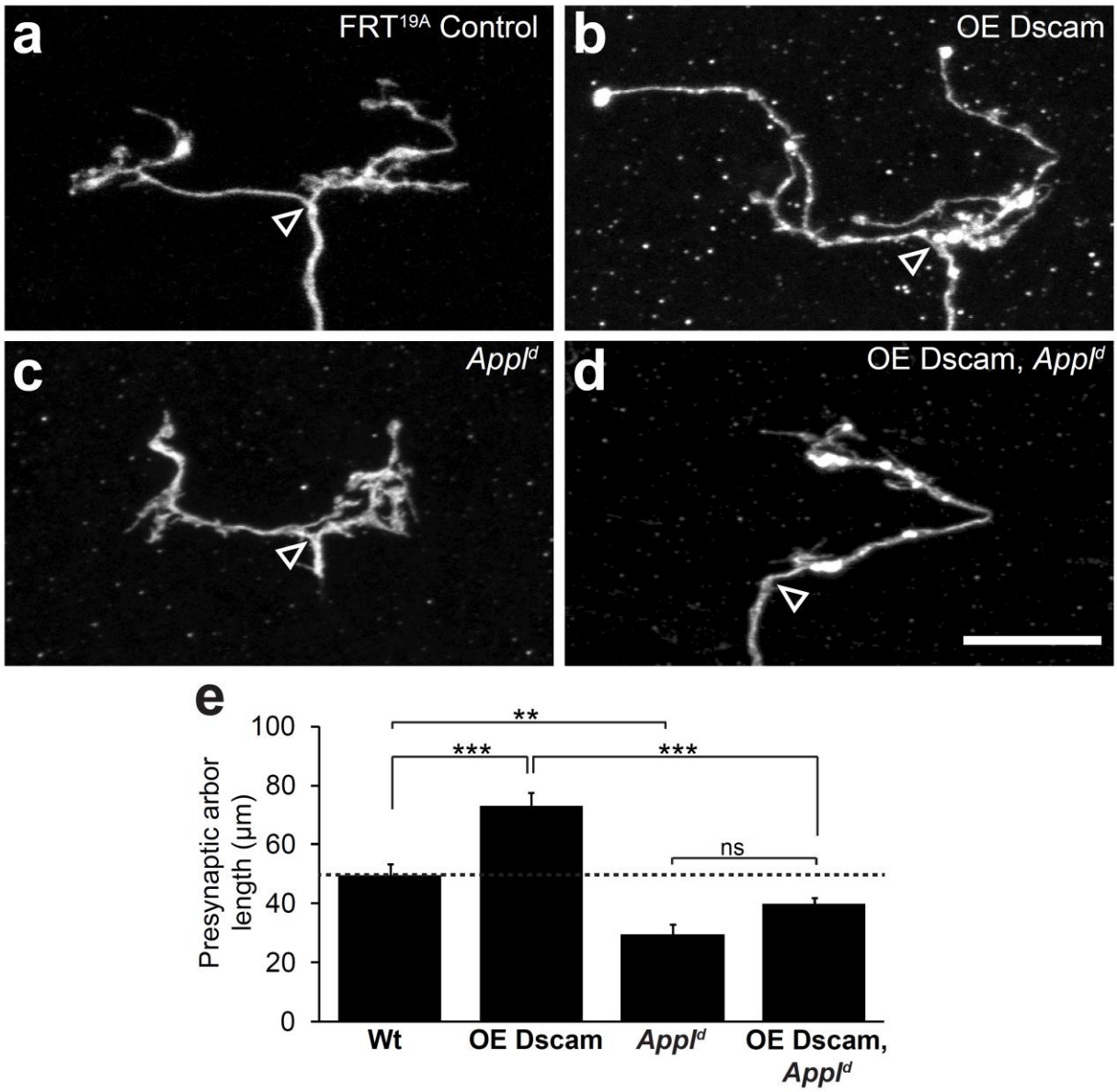

Figure 3.1 Dscam requires Appl to promote presynaptic arbor growth.
(a-d) Appl is requires in c4da neurons to instruct presynaptic arbor growth. The arrowhead shown in each panel points to the location where the axon elaborates the presynaptic arbor. While overexpression of Dscam::GFP (b) in single C4da presynaptic terminals leads to increased length when compared to control (a), overexpression of Dscam in *App*^{d} mutant neurons (d) leads to presynaptic terminal lengths that are not significantly different in length from *Appl*^{d} mutant neurons (c). However, *Appl*^{d} mutant neurons are significantly shorter than control neurons, suggesting that Appl is required to instruct presynaptic arbor growth. These effects are quantified in (e). Scale bar is 10µm.

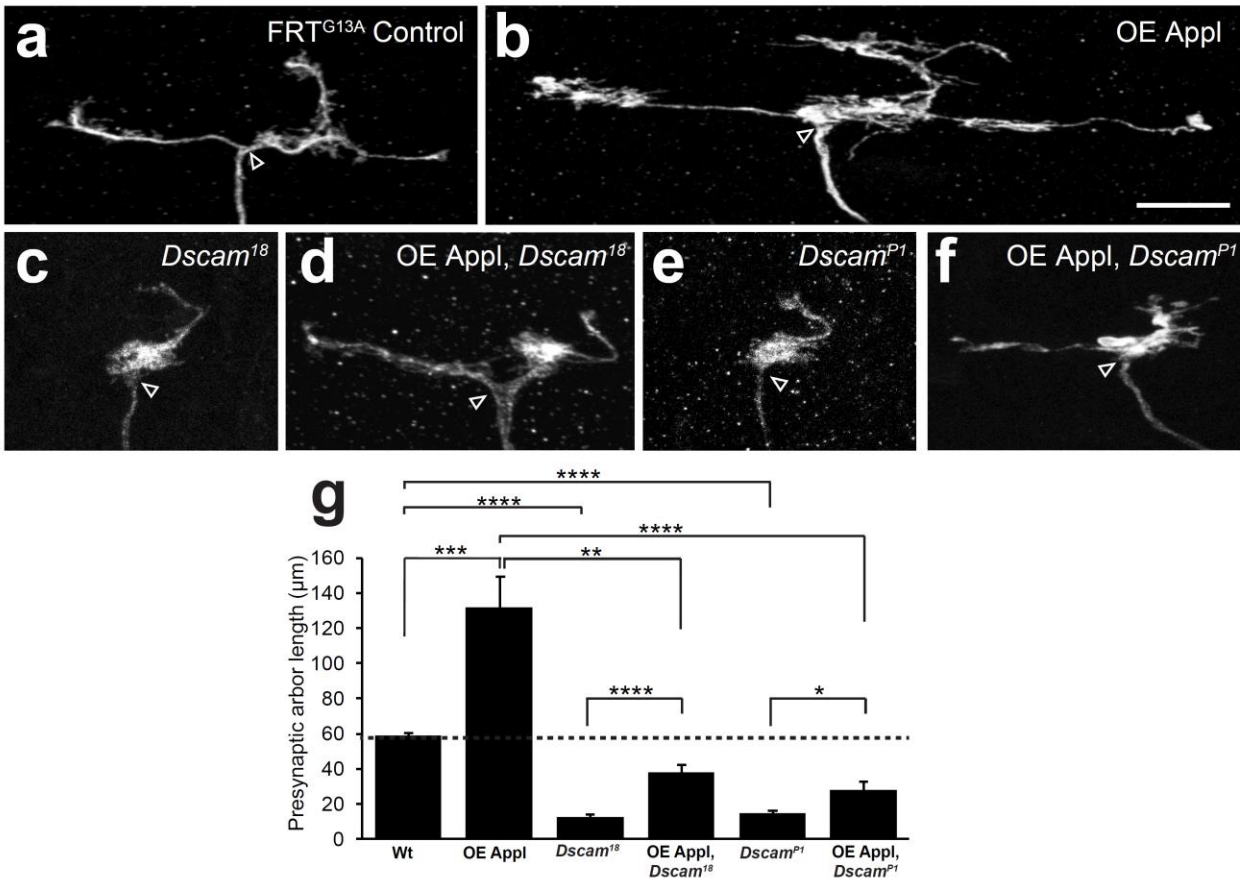

Figure 3.2 Appl requires Dscam to promote presynaptic arbor growth.
Like Dscam, overexpression of Appl in single C4da neurons leads to a significant increase in presynaptic arbor size (b) when compared to control clones (a). As previously reported, *Dscam*^{18} mutant neurons (c) have significantly shorter presynaptic arbors than control neurons (a). However, overexpression of Appl in *Dscam*^{18} (d) leads to presynaptic arbors than are significantly shorter than those that overexpress Appl in a wildtype background (b) but significantly longer than *Dscam*^{18} mutant neurons that do not have increased Appl . This effect was verified with an independent mutant allelle of *Dscam*, *Dscam*^{P1} (f and g) and is quantified in (g). Scale bar is 10 µm.

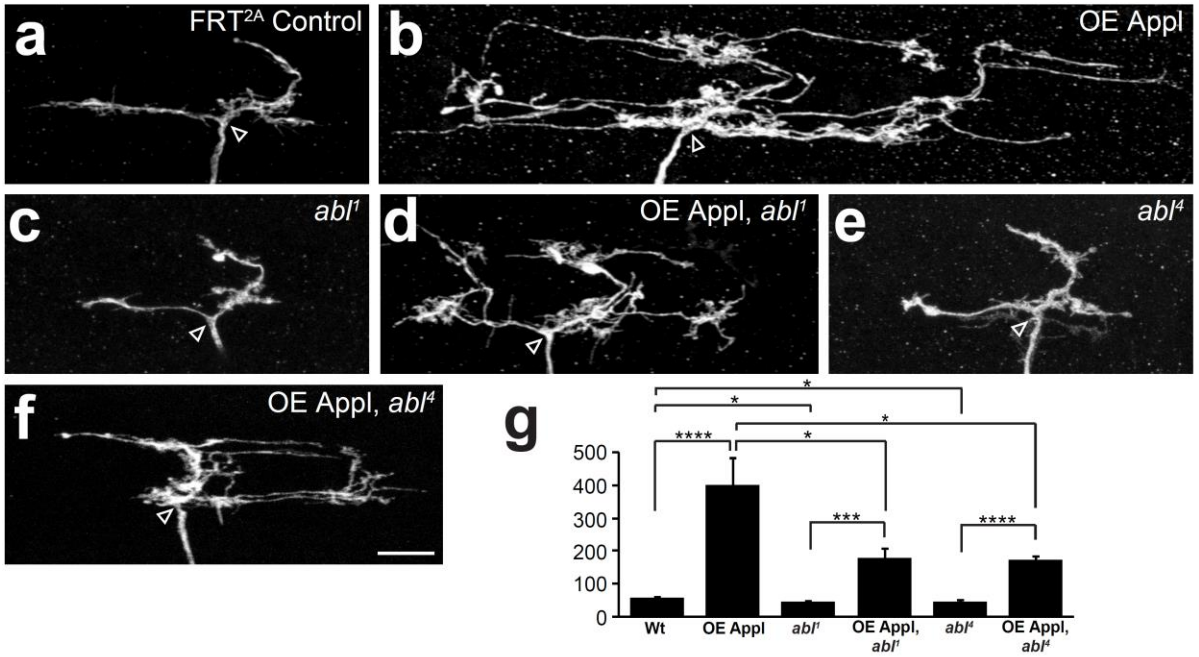

Figure 3.3 Appl requires Abl to promote presynaptic arbor growth.
Overexpression of another Appl construct in single C4da neurons (b) leads to very significantly increased presynaptic arbor lengths as compared to controls (a). As previously demonstrated, *abl*^{1} mutant C4da neurons have subtly but significantly shorter presynaptic arbors (c). Furthermore, overexpression of Appl in *abl*^{1} neurons leads to significantly shorter presynaptic arbors (d) when compared to overexpression of Appl in a wildtype background (b) , but significantly longer presynaptic arbor lengths than *abl*^{1} mutant neurons (c). This result was confirmed using an independent mutant allelle of *abl*, *abl*^{4} (e and f) and is quantified in (g). Scale bar is 10 µm.

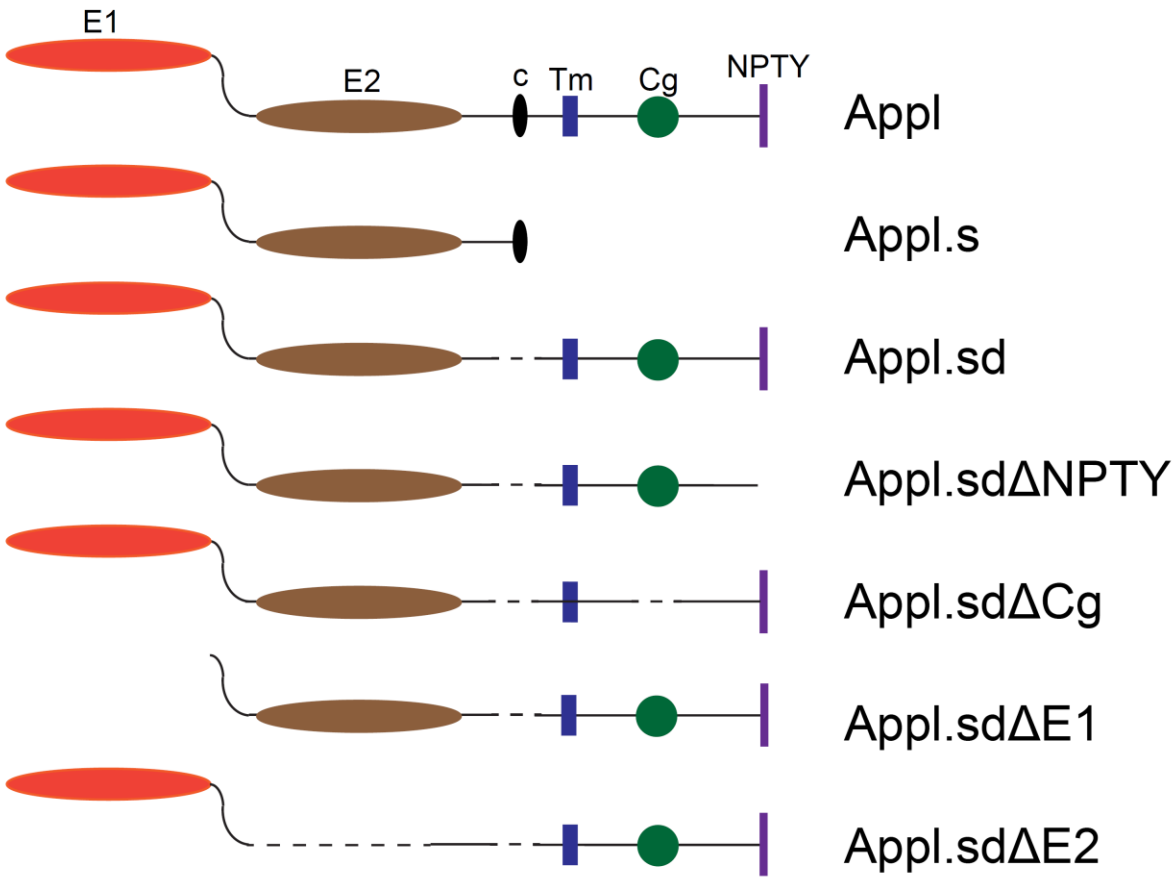

Figure 3.4 Appl deletion constructs used in this study.
Wildtype Appl contains two conserved cytoplasmic regions, E1 and E1, a cleavage site (c), a transmembrane domain (Tm), a conserved G_O-protein binding site (Cg), and a conserved internalization sequence (NPTY). Appl.s is a constitutively secreted form of Appl that lacks the transmembrane and cytoplasmic regions of Appl, while Appl.sd is a secretion deficient form of Appl that contains a short deletion that overs the cleavage site. Appl.sdΔNPTY lacks the GYENPTY internalization sequence and surrounding amino acids (872-883), Appl.sdΔE1 deletes the distal half of the E1 as well as a number of amino acids located on the c-terminal side of E1 (85-321 aa), Appl.sdΔE2 deletes 75% of the distal region of E2 as well as some amino acids c-terminal to it (449-740 aa), and Appl.sdΔCg,deletes the putative G_O-protein binding site (845-855 aa).

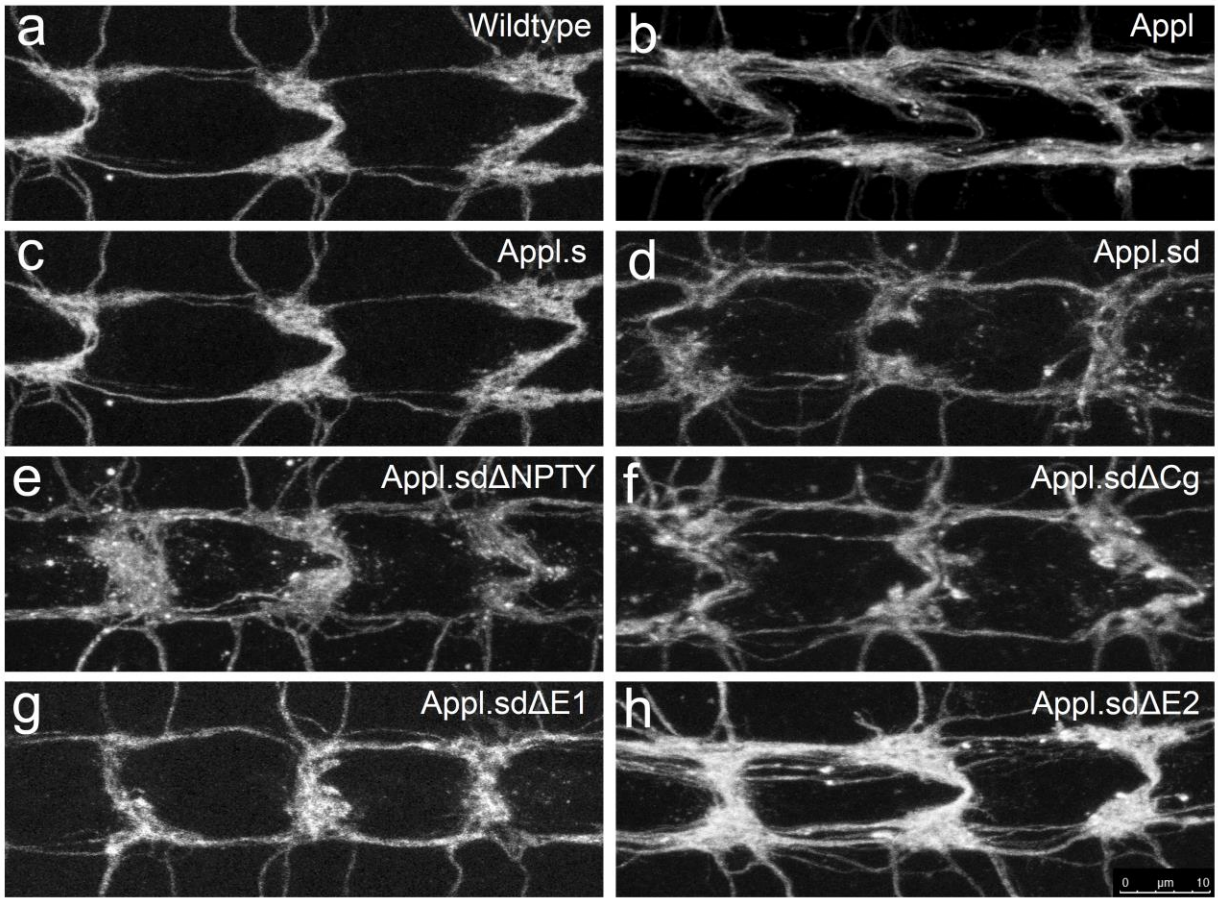

Figure 3.5 Analysis of Appl deletion constructs in presynaptic terminal growth.
In contrast to wildtype C4da axon ladders (a), C4da neurons that express wildtype Appl show exuberant presynaptic arbor growth (b). Conversely, overexpression of a consistutively secreted form of Appl (c) is indistinguishable from wildtype. As compared to C4da neurons that express Appl, C4da neurons that overexpress a secretion deficient form of Appl show an intermediate phenotype (d), with more presynaptic arbor growth than wildtype C4da neurons, but less growth than C4da neurons overexpressing wildtype Appl. When the evolutionarily conserved NPTY internalization site is further deleted from Appl.sd, an additional phenotype emerges. C4da neurons overexpressing Appl.sdΔNPTY have extremely convoluted and abundant processes in the neuropil region (e), suggesting that loss of the internalization sequence may hamper synapse formation. In contrast, when the conserved G_O-protein binding site is further deleted from the Appl.sd cytoplasmic domain (f), the phenotype is identical to Appl.sd. Appl.sd constructs with further deletions in two conserved extracellular regions have drastically different phenotypes form one another. Deletion of the E1 domain in Appl.sd completely abolishes Appl.sd's promotion of presynaptic arbor growth (g), while deletion of the E2 domain rescues it, such that C4da neurons that overexpress Appl.sdΔE2 (h) are indistinguishable from those that overexpress wildtype Appl. Scale bar is 10 µm.

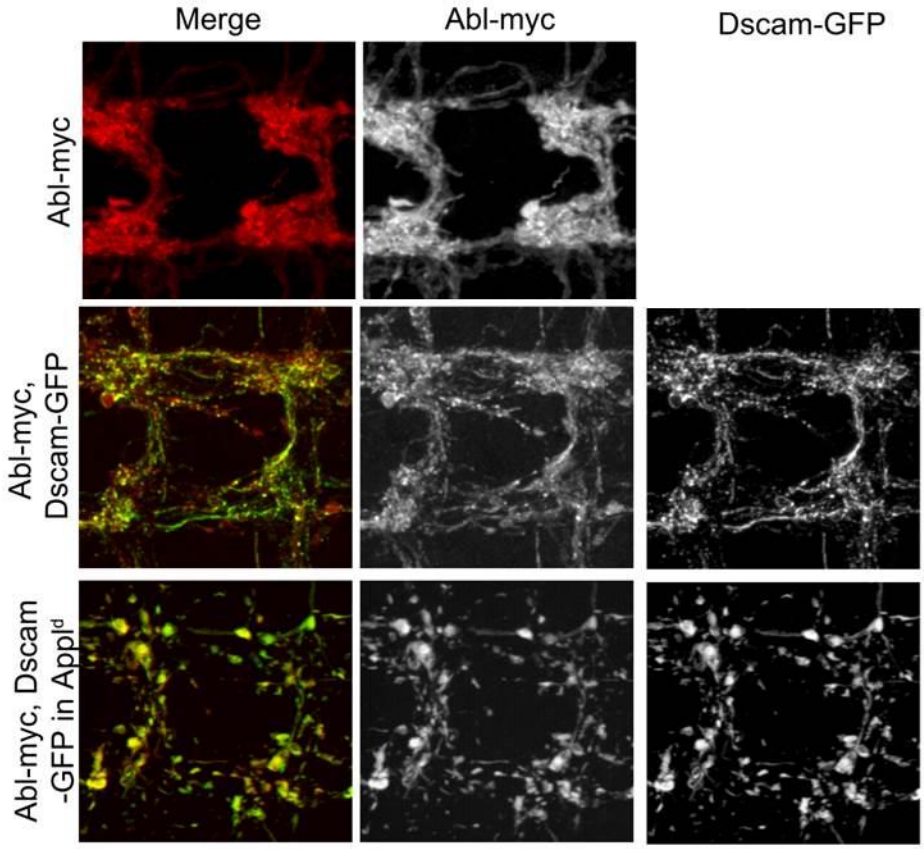

Figure 3.6 Appl is not required for Dscam and Abl colocalization in C4da presynaptic arbors in vivo.
Expression of Abl-myc in C4da presynaptic arbors leads to a uniform distribution of Abl-myc (red) throughout the presynaptic arbors (top row). Coexpression of Dscam-GFP (green) and Abl-myc leads to a redistribution of Abl-myc, such that Abl-myc becomes punctate and overlaps extensively with Dscam-GFP (middle row). Coexpression of Dscam-GFP and Abl-myc in an *Appl*^{d} mutant background leads to striking redistribution of Dscam-GFP and Abl-myc is also further redistributed, maintaining extensive overlap with the Dscam-GFP signal such that their patterns in the presynaptic arbors are nearly identical. These results suggest that *Appl* is not required required for in vivo binding of Dscam and Abl.

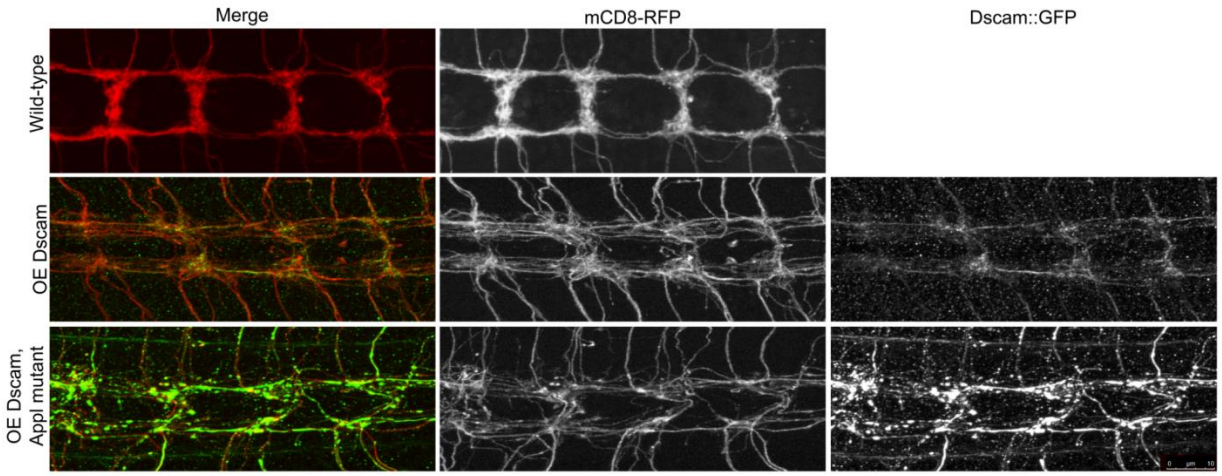

Figure 3.7 Loss of Appl leads to redistribution of Dscam-GFP in C4da presynaptic arbors. Overexpression of Dscam-GFP in C4da presynaptic arbors leads accumulation of small, dim Dscam-GFP (green) puncta throughout the presynaptic arbors, with slightly increased signal in the neuropil regions (middle row). In matched samples that were stained and mounted together as well as imaged simultaneously using identical imaging parameters, overexpression of Dscam-GFP in *Appl*^{d} mutant C4da presynaptic arbors (bottom row) led to redistribution of Dscam-GFP into extremely bright, large puncta. Dscam-GFP also formed long intense aggregations in the C4da axons as they entered the C4da presynaptic ladder region. Reduction in presynaptic arbor growth is also visible in C4da neurons overexpressing Dscam-GFP in an *Appl*^{d} mutant background using a membrane morphology marker, mcD8-RFP (red, center, bottom row) as compared to overexpression of Dscam-GFP in a wildtype background (center, middle row.

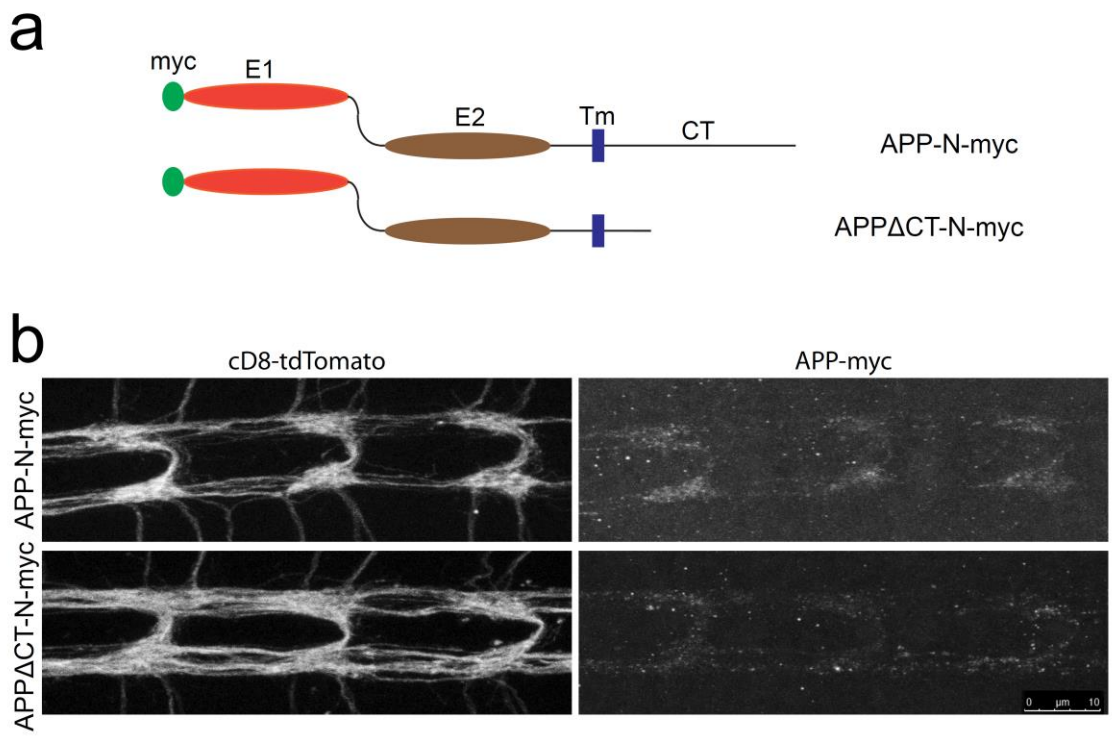

Figure 3.8 Overexpression of human APP695 promotes presynaptic arbor growth and does not require the cytoplasmic domain.
(a) Schematic showing APP-N-myc, which is full-length human APP695 with an N-terminal myc tag, and APPΔCT-N-myc, which is constructed as APP-N-myc but lacks the cytoplasmic domain. (b) Like *Drosophila* Appl, overexpression of human APP-N-myc also promotes presynaptic arbor growth (top row, left). Myc staining shows that APP-N-myc assumes a punctate pattern in the C4da presynaptic arbor ladder (top row, right). An APP construct that lacks a region of the cytoplasmic domain that includes the NPTY internalization sequence and the conserved kinesin-1 binding domain is expressed in C4da presynaptic arbors and retains its ability to promote presynaptic arbor growth (bottom row).

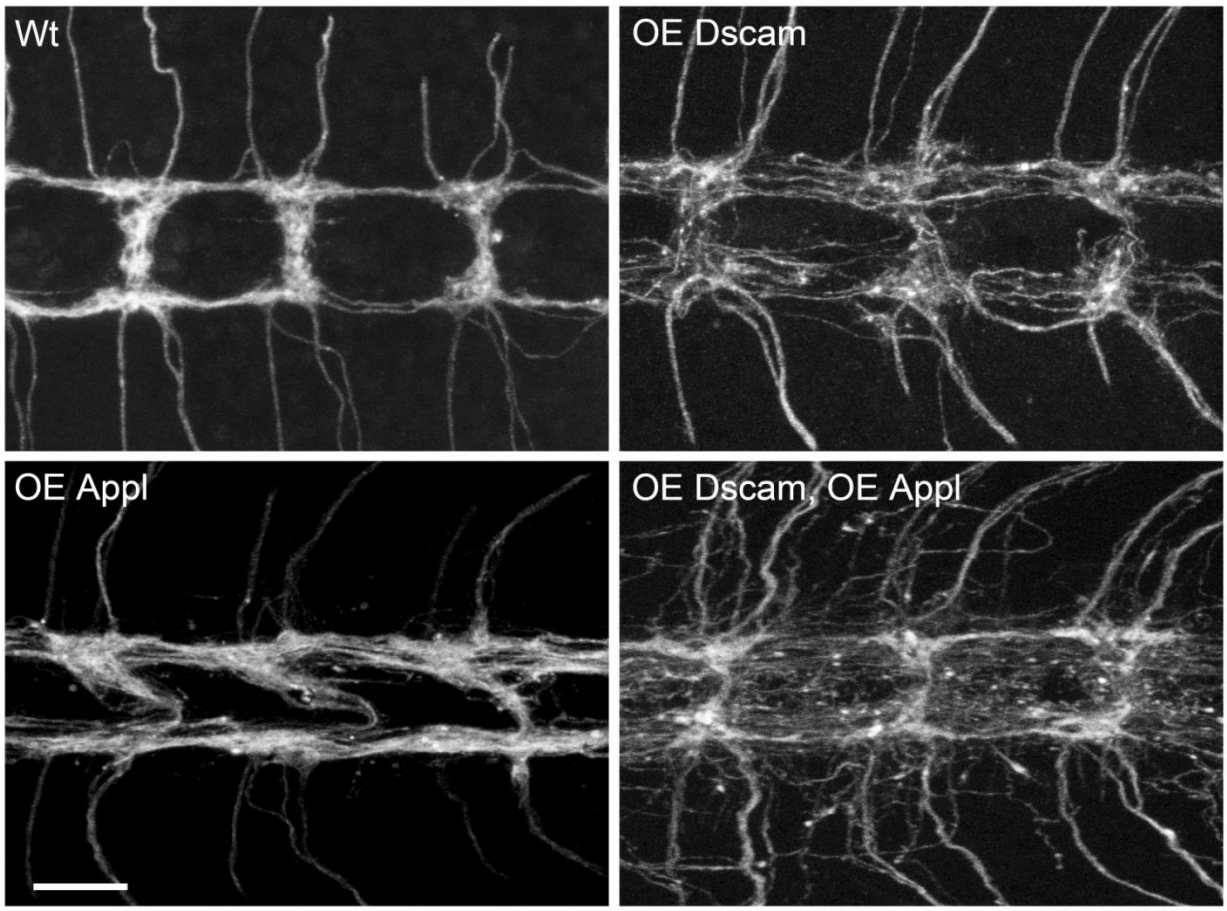

Figure 3.9 Coexpression of Appl and Dscam has a synergistic effect on C4da presynaptic arbor growth.
Overexpression of either Dscam (b) or Appl (c) alone leads to increased presynaptic arbor growth as compared to wildtype C4da neurons (a). However, both lead to presynaptic terminals that, while increased in size, generally remain within the confines of the C4da axon ladder (dashed red lines). In contrast, simultaneous overexpression of Dscam and Appl leads to wildly overgrowth presynaptic arbors that stay wildtype from the usual location of the C4da presynaptic arbor (d) (arrowheads).

3.6 Description of manuscript and authors' contributions

The experiments described in Chapter 3 of this dissertation have not yet been published at the submission of this dissertation. A manuscript is in preparation.

The writing, experiments, and figures in Chapter 3 of this dissertation were prepared and performed by GRS with direction from BY.

CONCLUSION

The results presented in this dissertation represent the first inroads into understanding the signaling mechanisms of Dscam in processes that are controlled by Dscam expression level. In particular, we show that Dscam requires Abl to promote presynaptic terminal growth and that Dscam-Abl binding and activation is mediated through the Dscam cytoplasmic domain both in culture and in vivo. Furthermore, we show that Dscam and Appl are mutually required, and that simultaneous upregulation of Dscam and Appl leads to a synergistic phenotype. This represents the first evidence that Dscam and Appl genetically interact and suggests that Dscam and Appl may act synergistically in the pathogenesis of Down syndrome and Fragile X syndrome. Here we discuss the pitfalls and limitations, possible future studies, and the potential of tyrosine kinase inhibitors as therapeutics for Down syndrome and Fragile X syndrome.

4.1 Pitfalls and limitations

4.1.1 Maternal contribution and mitotic perdurance of gene products in mutant analysis of gene functions.

Although extremely useful and powerful, the use of mutant analysis for studies of gene function during development is subject to two important limitations, maternal contribution and perdurance. During *Drosophila* oogenesis, the mother packs the oocyte with the RNAs and proteins that will be required for embryonic development prior to the zygotic transition, when the zygotic genome will be activated and the degradation of maternal products will commence. This

is known as maternal contribution. Thus, even an embryo that is homozygous mutant for a certain gene may contain protein and mRNA from that gene which has been contributed by the mother. Thus, an embryo can only be assumed to completely lack a protein of interested if the mother, too, is homozygous mutant for the relevant gene. Thus, homozygous mutant cells in some mutant embryos or larvae may still contain maternally contributed RNA and protein, which may, in turn, mask the phenotype caused by loss of that gene. We expect this to be the case of Abl, as discussed in Section 2.5. Furthermore, protein and RNA from a gene of interest may still be detected in a homozygous mutant neuron generated using MARCM even if that gene is not subject to maternal contribution. This can occur when gene products generated by the zygotic genome prior to the recombination event persist in the labeled cell. This is phenomenon is known as perdurance.

Although maternal contribution and perdurance are important to keep in mind when interpreting the results of mutant analysis, they rarely render results uninterpretable. In cases where maternal contribution in particular obscures the function of a gene in early development, the generation of maternal clones can reveal the mutant phenotypes that were masked.

4.1.2 Co-immunoprecipitation and colocalization analysis

The two techniques we used to show that Dscam and Abl interact with one another, co-immunoprecipitation and colocalization analysis, are limited in that they cannot tell us whether Dscam and Abl interact directly with one another. Although it appears that Dscam and Abl may at least interact within a common protein complex and that this interaction requires the Dscam cytoplasmic domain, it may be that another, yet unknown, adaptor protein or proteins acts as an intermediary between Dscam and Abl. The question of whether Dscam and Abl bind directly to one another thus remains open, but could be addressed using in vitro binding assays.

Colocalization analysis, as a technique, is further limited by the possibility that two proteins may appear to colocalize if they are located within the same cellular compartment, even if they do not interact. We believe that the results presented here (see Figure 2.5) are informative due to the fact that loss of the Dscam cytoplasmic domain abolishes Dscam-Abl colocalization. However, this particular technique should be used with caution and in conjunction with other, complementary assays.

4.1.3 Western blotting using S2 cells

All of the western blots presented in this dissertation, with the exception of that in Figure 2.7d, were performed in S2 cells using overexpression of tagged proteins. Thus, when interpreting these results, it is important to keep in mind that S2 cells are not neurons. S2 cells were derived from primary culture of late stage 20- to 24-hour *Drosophila* embryos and present a macrophage-like lineage (Schneider, 1972). Therefore, experiments done in S2 cells may not reflect the environment that would be seen in neurons or in their presynaptic arbors. However, our in vivo results corroborate each of the conclusions that were derived from S2 cell experiments, suggesting that S2 cells may serve as a useful initial system in which to test molecular hypotheses before commencing in vivo investigations.

Another caveat to our S2 cells experiments and some of our in vivo experiments is that they were performed using overexpression of tagged *Drosophila* proteins. Although it would be ideal to use endogenous proteins, the availability of antibodies that have been raised to recognize *Drosophila* proteins is limited. Furthermore, endogenous proteins may not be expressed at high enough levels to be detected using our methods. A risk of using overexpression is the possibility of detecting artificial interactions between proteins that result from the high level of expression in the system. We believe that this is not the case in our experiments, however, owing to the fact

that Dscam and Abl appear to genetically interact in in vivo assays that do not rely on transgene overexpression (see Figure 2.1 l, m, n, and p). This cannot be said of the genetic interactions we show between Appl and Dscam, however. Future studies should seek to confirm genetic interactions between Appl and Dscam without the use of transgenes. However, even if such an interaction is not found, these studies are still useful to understand the function of Dscam and Appl in situations where their expression levels are increased, as in Down syndrome or Fragile X syndrome. To reduce expression to levels that might be seen in human patients with these disorders, future studies might use BAC transgenics to increase Dscam or Appl copy number or focus on *dFMRP* mutants.

4.2 Future work

With Dscam, Appl, and Abl as a starting point, future work should focus on expanding our understanding of Dscam and Appl signaling. As demonstrated here using Abl, each newly identified pathway member represents a novel potential therapeutic target. Two unresolved questions in the work presented here are: what is the mechanism by which Dscam and Appl interaction and which molecules link Abl to the cytoskeleton after activation by Dscam. A number of experiments laid out earlier have the potential to answer the first question by differentiating between the hypotheses that Appl controls Dscam trafficking and that Appl and Dscam act as co-receptors. The second question may be addressed by first investigating the roles of molecules that have previously been shown to interact with Abl, including *Enabled* (Comer et al., 1998), *Trio* (Hill et al., 1995), and *Rac1* (Singh et al., 2010). Further interactors with Dscam and Appl may also be identified by using a candidate approach to further illuminate the roles of molecules that have previously been implicated in Appl's signaling in mushroom body neurons

and motor neurons. These include *Frizzled*, *Flamingo*, *Van Gogh*, *Disheveled* (Soldano et al., 2013), and G_O-protein (Torroja et al., 1999b).

We describe here a proof-of-concept experiment that presents tyrosine kinase inhibitors as a potential therapy for disorders in which Dscam expression is increased. Since Appl also partially requires Abl to promote presynaptic terminal growth, we hypothesize that tyrosine kinase inhibitors may also mitigate the consequences of simultaneous Dscam and Appl upregulation. However, future experiments are required to validate this hypothesis.

4.3 Potential of tyrosine kinase inhibitors as therapeutics for brain disorders

One exciting outcome of this work is the potential to use tyrosine kinase inhibitors like nilotinib to treat disorders in which Dscam expression is elevated, such as Down syndrome and Fragile X syndrome. However, many questions remain before this strategy can be tested in human patients. Importantly, we determined the morphological consequences of increased Dscam expression in *Drosophila* neurons. Whether the observed changes in neuronal morphology lead to behavioral alterations and deficits in *Drosophila* is still unknown. However, changes in neuronal morphology are observed in both Down syndrome and Fragile X syndrome, suggesting that morphological alterations may be causative for intellectual disability in each of these disorders (Kaufmann and Moser, 2000). Furthermore, whether treatment with tyrosine kinase inhibitors can rescue the behavioral changes seen in *Drosophila dFMRP* mutants, a fly model of Fragile X syndrome, remains to be tested. If treatment with nilotinib can rescue the learning and memory and circadian rhythm phenotypes that characterize this model, it would suggest that inhibiting tyrosine kinase activity may prove therapeutic for brain disorders with increased Dscam expression.

In addition to unknowns in the *Drosophila* system, the question of whether the Dscam-Abl pathway is conserved in mammals has yet to be answered. This question can be relatively quickly answered by using mammalian cell culture to test whether mammalian Dscam and Abl bind to one another and whether mammalian Dscam activates Abl kinase activity in vitro. If these properties are conserved, the next step would be to determine whether the Dscam-Abl pathway participates in the development of presynaptic arbors in mammals. In this vein, it is interesting to note that Abl has been shown to be required for postsynaptic development of the NMJ in mice, where it is thought to signal through MuSK to cluster AChRs on the post-synaptic myotubule (Finn et al., 2003). Developing a system to assess presynaptic arbor size control in the mammalian central nervous system will prove useful for this line of study.

In the event that Dscam and Abl are found not to be involved in mammalian presynaptic arbor development, it is equally possible that the Dscam-Abl pathway is conserved in mammals but mediates different aspects of development. For example, Dscam mutant mice have a variety of neurological phenotypes, including severely uncoordinated gait, spontaneous seizures and kyphosis. In addition, Dscam mutant mice exhibit retinal abnormalities, including defective laminar specificity, neurite arborization, and cell body spacing (Fuerst et al., 2008). Thus, it is possible that Abl mediates Dscam's function in any of these developmental roles. Furthermore, several mouse models of Down syndrome, like Ts16, Ts1Cje, and Ts65Dn, have three copies of the Dscam gene and have a host of neurological phenotypes that have been well characterized (Seregaza et al., 2006). Although it is not yet established which, if any, of these phenotypes are the result of increased Dscam expression, this question may be asked by reducing copy number of Dscam in these model systems by introducing a single copy of a loss-of-function mutation of

Dscam. If any phenotypes caused by increased Dscam expression are discovered, it could then be asked whether Abl was required to mediate Dscam's role in these processes.

Finally, it may be possible to test the utility of tyrosine kinase inhibitors as a potential therapy for Down syndrome and Fragile X syndrome by directly treating mouse models of these disorders with tyrosine kinase inhibitors and evaluate the effects on cognition and behavior. Although studies of this kind would not shed light on the mechanism of action, they would indicate whether treatment with tyrosine kinase inhibitors might have utility for mitigating the cognitive and behavioral consequences of these disorders. The neurological phenotypes of a number of mouse models of Down syndrome that include three copies of Dscam have been well documented with established behavioral assays, including learning and memory using the Morris water maze and fear conditioning tasks, and grip strength (Costa et al., 2010). Furthermore, the mouse model of Fragile X syndrome, *FMR1* mutant mice, has been shown to have deficits in learning and memory using the Morris water maze (Consorthium, 1994) and sensory hypersensitivity using the prepulse inhibition paradigm (Chen and Toth, 2001). Thus, it may be informative to treat these mouse models with tyrosine kinase inhibitors and assess changes in learning and memory, grip strength, and sensory sensitivity using established assays. If tyrosine kinase inhibitors positively impact the behavior of these mouse models, it will set the stage for clinical trials in humans.

One of the unique advantages to using tyrosine kinase inhibitors to treat developmental disorders is that a number of tyrosine kinase inhibitors, including imatinib and nilotinib, are already approved by the FDA for use in humans, though for treating leukemia. In addition, patients with chronic myeloid leukemia may take imatinib or nilotinib continuously for a period of years without significant adverse effects(Druker, 2008). However, several unknowns about the

feasibility of treating developmental disorders with tyrosine kinase inhibitors remain, including whether they are safe for long-term use in pediatric patients. However, several safety studies in pediatric patients have already been completed, with promising results. Dasatinib, which is known to cross the blood-brain barrier (Porkka et al., 2008), has shown similar efficacy and side effects as have been noted in adults in pediatric patients treated for less than 1 month up to 50.6 months. Like adults, the most common side effects were nausea, headache, diarrhea, and vomiting. Importantly, no drug-related deaths were observed (Aplenc et al., 2011; Zwaan et al., 2013). Nilotinib, which was used in our experiments and is known to cross the blood-brain barrier (Hebron et al., 2013), is currently being studied in Phase I and Phase II trials which aim to assess the safety and efficacy of treating pediatric chronic myeloid leukemia patients. In the meantime, compassionate use studies and case reports suggest that nilotinib has a similar safety profile in children as in adults (Wayne, 2008). Thus, existing studies suggest that tyrosine kinase inhibitors are likely to be safe for pediatric patients, but further work remains to confirm the safety of nilotinib.

If tyrosine kinase inhibitors are deemed safe for administration to pediatric patients, another challenge for validating these drugs for treating Down syndrome and Fragile X syndrome is determining the optimal treatment window. Though neurological abnormalities in Down syndrome and Fragile X syndrome are thought to begin prenatally, treatment in utero is unlikely to be an option due to the documented teratogenicity of tyrosine kinase inhibitors in mammals. Teratogenicity and fetal loss have been documented in rats and rabbits administered imatinib and dasatinib. Although administration of nilotinib did not lead to teratogenicity, studies of rats and rabbits indicate a low absorption of nilotinib in the fetus, which could explain the lack of teratogenicity (Abruzzese et al., 2014). Low absorption of nilotinib by the fetus may also

explain the lack of observed teratogenicity in human patients who continued treatment with nilotinib during accidental pregnancies (Conchon et al., 2009; Mseddi, 2012). Though tyrosine kinase inhibitors are unlikely to be safe if administered prenatally, postnatal treatment may still prove effective. At the time of birth, human cognitive development is still progressing at a rapid pace and continues until approximately the onset of puberty (Chugani et al., 1987). Although evidence in humans is limited due to the dearth of healthy human tissues for study and of patients willing to subject their healthy children to PET imaging, However, limited data suggests that synaptogenesis peaks between the ages of 2 and 5 years (Huttenlocher and Dabholkar, 1997; Liu et al., 2012). Thus, we believe that treatment with tyrosine kinase inhibitors is likely to provide greatest benefit during this critical period of rapid synaptogenesis. Whether treatment beyond this 2 to 5 year-of-age period would be likely to prove therapeutic is difficult to speculate about. This question might be addressed first in *Drosophila* or mouse models of Down syndrome or Fragile X syndrome, should tyrosine kinase inhibitor treatment show efficacy in treating behavioral defects in these models.

Despite the many unknowns that remain to be investigated before clinical trials for tyrosine kinase inhibitors in Down syndrome and Fragile X syndrome patients commence, we believe that this strategy, at present, shows promise as a mechanistically-relevant treatment for these disorders.

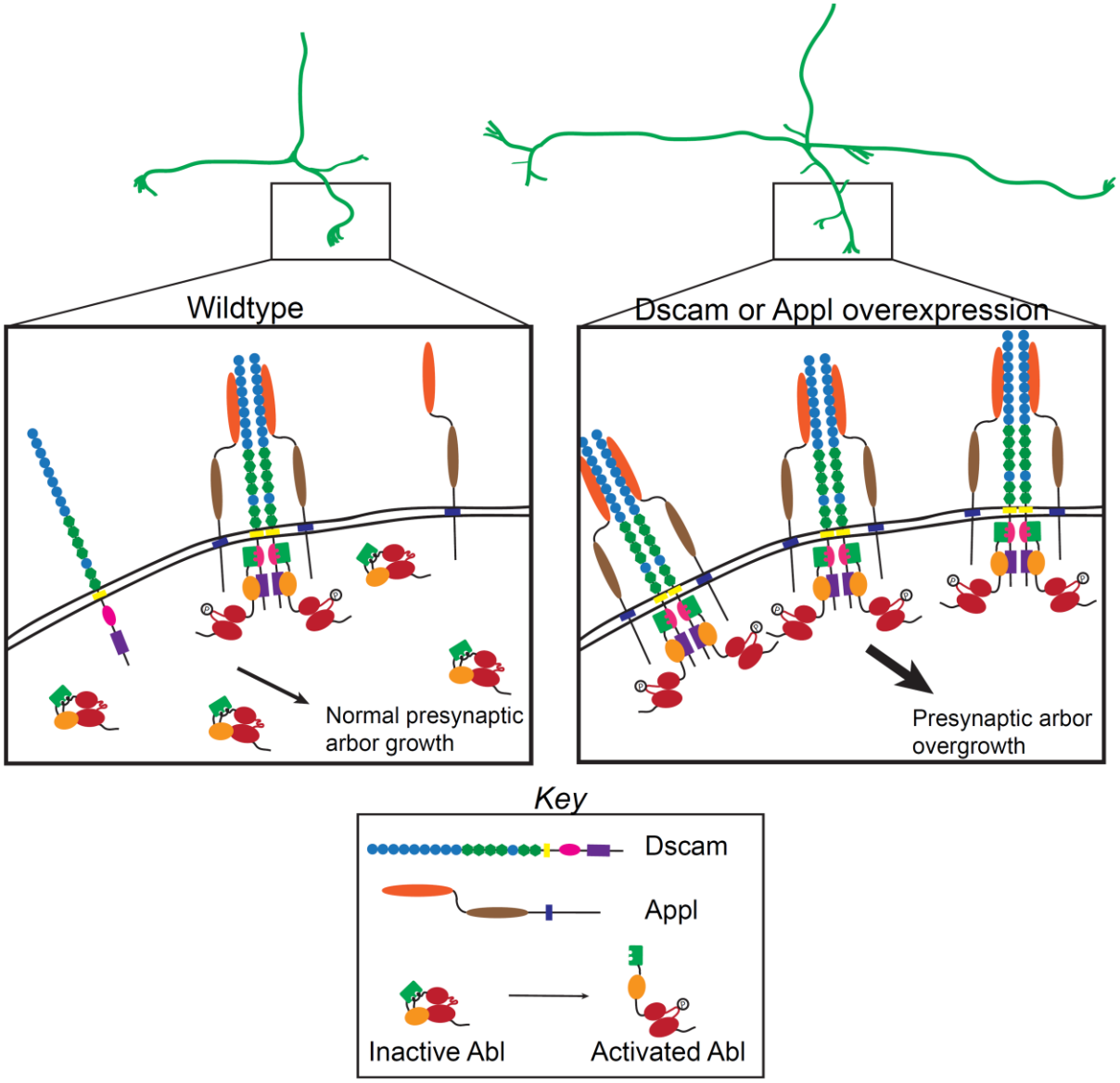

Figure 4.1 Summary of Hypotheses.
My current working hypothesis based on the experiments presented in this dissertation is that Dscam and Appl act as coreceptors to activate Abl kinase. In wildtype animals (left), normal Dscam and Appl levels mean that some Dscam bind to some Appl, which in turn leads to activation of Abl through Abl binding to the Dscam cytoplasmic domain. This, in turn, leads to normal presynaptic arbor growth through yet unknown downstream mechanisms. However, in conditions like Down syndrome or Fragile X syndrome where Dscam and Appl expression is increased, more Dscam and Appl means increased Dscam-Appl binding, which leads to more Abl binding to the Dscam cytoplasmic domain and becoming activated. More active Abl then leads to increased presynaptic arbor growth through yet unknown downstream events.

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